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13 ABSTRACT (Maximum 200 words)

Hydrogen cyanide reacts with α,β-unsaturated ketones (enones) to form stable compounds under physiological conditions (temperature, pH). Rate enhancement by the presence of a suitable catalyst would permit the use of enones as prophylactic drugs for cyanide exposure. An enone drug was synthesized and shown to have low acute toxicity. A transition state analog for the reaction of the drug with cyanide was designed, synthesized, and conjugated to protein. The conjugate was used to produce over 300 hybridomas producing antibodies that bind the TSA. Four monoclonal antibodies significantly enhanced the reaction rate, as shown by HPLC analysis of the product. The TSA markedly diminished the reaction velocity, indicating the involvement of the antibody binding site in the observed enhancement. Kinetic analysis of one antibody gave values of K_{enone} and K_{KCN} of 51 μM and 9.6 mM, respectively, with a value of k_{cat} of 2.33 h^{-1}. Computer simulations showed that (among other combinations) reducing K_m for both reactants by a factor of 10 and increasing k_{cat} by a factor of 600 would produce a practical antibody for cyanide detoxification. To accomplish this, we recommend an approach using modified TSAs to produce improved antibodies combined with protein engineering techniques to further enhance activity.

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PI - Signature C. Edgar Cook DATE 5/25/93

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1.0 Introduction

1.1 Background

The toxicity of cyanide and its ready availability make the development of treatment or prophylactic regimens of importance. Current treatment methods include:

- Administration of drugs that bind cyanide,
- Administration of drugs that induce methemoglobin formation with the resulting methemoglobin binding cyanide, and
- Administration of drugs that augment endogenous enzymatic detoxification.

An extension of the last method would be administration of both a drug that reacts with cyanide and an enzyme that catalyzes the reaction. For example, administration of the enzyme rhodanese in combination with thicsulfate has been shown to increase protection against cyanide (relative to thiosulfate alone) in laboratory animals (Frankenberg, 1989). The enzyme could be replaced with a catalytic antibody. The antibody could be administered prior to anticipated exposure to cyanide, because of the relatively long half-life of antibodies. The drug could be administered prophylactically as long as the threat existed.

The concept of catalytic antibodies is that an antibody to the transition state of a reaction would be able to catalyze the reaction just as an enzyme can--by stabilizing the transition state and thus lowering the energy required for the reaction. Catalytic groups (for example, acid-base catalysts) can also play a role.

To develop an antibody against the transition state of a reaction, one must design an analog that is stable but is close in structure to the transition state. When this compound is conjugated to a protein, the resulting antigen can stimulate the formation of antibodies which stabilize the transition state.

Numerous reviews of catalytic antibodies have been published (inter alia, Lerner et al., 1991; Skokat and Schultz, 1991; Baron, 1992; Benkovic, 1992). The first and simplest example of catalytic antibodies involved ester hydrolysis. The transition state for ester hydrolysis resembles structure **A-2** (Chart A). The trigonal carbonyl carbon of ester **A-1** becomes tetra-

hedral before collapsing to yield alcohol and carboxylic acid. Thus tetrahedral phosphonate esters (**A-3**) are transition state analogs (TSAs) for ester hydrolysis. Antibodies formed to phosphonate esters have been shown to catalyze ester hydrolysis, with more than 20 such reactions having been reported. Rate accelerations of nearly 10^8 M compared with uncatalyzed reactions have been observed. At least 17 different types of organic reactions had been reported to be catalyzed by antibodies by 1991 (Lerner et al., 1991). Although the development of antibodies which catalyze bimolecular reactions, such as amide bond formation, has been achieved, this task is more difficult than developing antibodies to catalyze unimolecular or hydrolytic reactions. To our knowledge antibodies catalyzing 1,4-additions to simple conjugated ketones have not been reported, although catalyzed addition of nucleophiles to 6-hydroxyxanthen-3-one has been demonstrated (Janjic and Tramontano, 1990).

1.2 Approach

To apply the catalytic antibody-drug approach to the removal of cyanide requires:

- Selecting a reaction of cyanide;
- Defining the transition state of the reaction;
- Selecting a structural analog that approximates the transition state;
- Designing an appropriate drug;
- Designing the TSA in a form that can be linked covalently to protein;
- Synthesizing the drug, TSA, and protein conjugate;
- Developing monoclonal antibodies to the TSA;
- Screening the antibodies to find one which catalyzes the desired reaction; and
- Biological testing.

Cyanide reacts with enones by the pathway shown in Chart B. Study of the kinetics of the process (Agami et al., 1982) indicates that the transition state is product-like (i.e., it resembles structures **B-3** and **B-4**). One approach to a TSA would be to stabilize the enol form **B-4** by forming a stable enolate, such as an enol ether, an enol silyl ether or perhaps an

enol phosphate, phosphonate, or phosphinate (**B-6**). Alternatively, one could mimic the enolate form **B-3** by means of a nitrone.

Once the basic transition state mimic has been defined, the complete structure of the hapten used for conjugation to protein must be designed, together with the structure of the drug which is to react with cyanide under antibody catalysis. The structure of the product of this reaction must also be considered. The structures of the hapten (**C-1**), drug (**C-2**), and product (**C-3**) combination developed are shown in Chart C.

We considered that it would be important to have the relative affinities for catalytic antibody to be drug < transition state (analog) >> product. The increased affinity on going from drug to transition state would lower the activation energy. The product should have lower affinity than the drug to prevent product inhibition of the reaction.

The position of the double bond in **C-1** mimics the transition state in which the cyanide would already be almost bonded. The oxygen enolate of **C-1** can help to develop a hydrogen-bond donor site in the antibody to further stabilize the transition state.

Aromatic moieties (e.g., phenyl) should enhance immunogenicity. Because their steric positions would shift during the reaction, differential binding should be enhanced. Since α-aryl enones react more rapidly with cyanide than do β-arylenones (Nagata and Yoshioka, 1977), the α-position is preferable for the aryl group. Also, the intrinsic reactivity can be controlled by placing electron-withdrawing or electron-donating substituents on the α-aryl group. Enone compounds such as the chalcones are not mutagenic. Thus although the toxicity of the selected drugs is subject to experimental findings, a priori low toxicity may be expected.

An alkyloxy link to the aryl group was chosen to separate the hapten from the protein in the immunogen. A terminal hydroxyl group provides a means for linking to protein. It also can be acylated or alkylated to provide drug structures with varying degrees of lipophilicity.

2.0 Contract Summary

2.1 Approach

The toxicity of cyanide and its ready availability make the development of treatment or prophylactic regimens of importance. The overall purpose of this project was to demonstrate a new approach to preventing or reversing the toxicity of cyanide by use of a drug plus catalyst combination. The drug would be selected from compounds which react with cyanide. The catalyst would be an antibody to the transition state for the reaction. The purpose of the catalyst would be to enhance the reaction rate so that the drug/cyanide reaction became fast enough to provide protection against cyanide intoxication. Although the specific purpose of this study was to devise such a combination for cyanide, the concept is more general and could in principle be applied to other toxic materials, e.g., as a chemical defense against mustard or neurotoxin attacks. It is related to one current method for cyanide treatment–the administration of drugs such as thiosulfate which augment endogenous enzymatic detoxification. However, since catalytic antibodies can be developed to enhance reactions for which there is no known enzyme available, the combination of drug plus catalytic antibody provides additional flexibility to the development of treatment modalities.

To carry out this work, we selected the reaction of cyanide with α,β-unsaturated ketones (enones). Since there was reasonable information about the transition state for this reaction, this permitted us to select a reasonably stable structural analog which approximated the transition state for the reaction of a specific enone (the drug), prepare this TSA in a form that could be covalently linked to protein, use the protein to immunize mice by hybridoma fusion techniques to produce a panel of monoclonal antibody, screen the antibodies to find those which catalyze the reaction of the enone with cyanide and carry out kinetic experiments designed to characterize the catalytic reaction.

2.2 Uncatalyzed Kinetics of Hydrogen Cyanide Addition to Enones

The addition to HCN to enones is dependent upon the concentration of cyanide ion present in solution and is first order in both [CN^-] and [enone]. Since HCN is a weak acid, the

concentration of cyanide ion will decrease with decreasing pH. Therefore a number of experiments were carried out with enones under physiological conditions (pH 7.4 and 37°C) to demonstrate that the uncatalyzed reaction would proceed at a measurable rate under these conditions. [Other electron-deficient, double-bond systems such as α,β-unsaturated nitriles and α,β-unsaturated esters also undergo addition of HCN in a similar manner. Preliminary experiments suggested that, in accord with the literature (Nagata and Yoshioka, 1977), the reactivity of unsaturated ketones was somewhat greater.] The reaction of 4-phenyl-3-buten-2-one with cyanide to produce 4-cyano-4-phenylbutane-2-one was studied kinetically. Disappearance of starting enone and appearance of product cyano ketone were measured by HPLC, and the second order rate constant under these conditions of pH and temperature was about 0.025 $M^{-1}h^{-1}$ for the reaction in buffer containing a small amount of acetonitrile. Computer simulation studies indicated that an overall rate enhancement of this particular reaction of about 3×10^6 would be required to make it therapeutically useful. Rate enhancements nearing 10^8 M have been observed in reactions catalyzed by antibodies. Furthermore, kinetic simulations using a projected model of the catalytic reaction showed that, with kinetic association and dissociation rate constants for binding within the ranges usually associated with antibodies (Nisonoff et al., 1975) and with rates which are consistent with those of some of the better catalytic antibodies, pharmacologically relevant concentrations of cyanide and drug could undergo catalytic combination in a time rapid enough to give protection against the toxic effects of cyanide. The dose of antibody required to fulfill these conditions (140 mg/kg) is well within the range of antibody treatments given to humans and also well below the dose of 7500 mg/kg of Fab antibody fragments given intravenously to rats over a 1 h period without apparent toxicity (Pentel et al., 1988).

Several enone/TSA combinations were investigated for attempted synthesis during the course of this work. Computer-assisted molecular modeling studies were used to indicate that the topographical features of the enone, TSA and product were such that the product would be expected to have a lower affinity for antibody derived from the TSA than would the enone

The enone structure was similar enough to that of the TSA to anticipate that binding to the antibody could occur but with lower affinity than the presumed transition state. Since an energy differential contributes to the stabilization of the transition state, this order of affinities was projected to be the desirable one. Because of the ability of the small molecules to achieve a number of conformations at relatively low energy cost and because of the potential flexibility of the antibody molecule, such computer modeling studies are indicative but not definitive.

2.3 Synthesis of Drug, TSA, and Immunogen

The initially designed drug was based on a cyclohexenone structure, which permitted certain desirable properties to be designed into the system. Extensive synthetic studies showed that the initial routes, although based on reasonable analogous preparations in the chemical literature, could not be applied to the preparation of the desired 2-aryl-3-methylcyclohex-2-ene-1-ones. Therefore, two new synthetic routes were developed to compounds of this general structure, and a desired 2-(substituted naphthyl)-3-methyl-cyclohex-2-ene-1-one was prepared. Numerous attempts to convert this to a stabilized enol methyl ether analog were unsuccessful. The trimethylsilyl (TMS) enol ether was prepared and characterized but was unstable under the conditions required for conjugation to protein. Attention was given briefly to the preparation of appropriate 3-arylcycloenones and 1,3-aryl-2-propene-1-ones (chalcones) before selecting a 1,2-diaryl-2-propen-1-one (compound **C-2**, Chart C) as the drug. This compound was readily prepared by alkylation of the phenolic OH of 4-hydroxyphenylbenzyl ketone with 3-bromopropanol followed by Mannich reaction with formaldehyde. It was converted to the cyano ketone product **C-3** with KCN in the presence of ammonium chloride. A combination of steps including O-alkylation of a potassium enolate of this compound gave the desired enol ether (a methoxystilbene) which was conjugated to bovine serum albumin (BSA) and bovine thyroglobulin (BTg) by means of an activated urethane intermediate (**C-1c**).

2.4 Immunization of Mice and Testing of Sera

Three groups of BALB/c mice were immunized with the methoxystilbene TSA coupled to carrier protein. One group (Group 1) was immunized with TSA coupled to BTg (TSA-BTg) and the other two groups (Group 2, Group 3) with TSA coupled to BSA (TSA-BSA). For immunization, mice were given a primary injection (Day 0) of 50 µg of immunogen emulsified in complete Freund's adjuvant and administered intraperitoneally. Booster injections were given on Days 28 and 42, and every 2 weeks thereafter until termination of the immunization protocol. Booster injections consisted of 25 µg of immunogen emulsified in incomplete Freud's adjuvant and administered intraperitoneally. The immunization schedule for groups 1 and 2 is given in Table I, and the schedule for group 3 is given in Table II.

Fourteen days after the primary immunization and 7 days after the booster injections, a small volume of blood was collected from each mouse, and the serum was tested by enzyme immunoassay (EIA) for the presence of antibodies to the TSA.

As shown in Table III, most of the 12 mice immunized with the TSA-BTg conjugate had moderate levels of serum antibodies 14 days after the primary immunization (Bleed 1). In contrast, only 5/24 mice immunized with the TSA-BSA conjugate had detectable serum antibodies at that time. However, 7 days after the first booster injection (Bleed 2), almost all mice had moderate-to-high levels of serum antibodies.

Sera from Bleed 2 from all mice were tested in competitive inhibition enzyme immunoassays (CIEIA). Results for groups 1 and 2 are shown in Figure 1; similar results were obtained for group 3. In the presence of free TSA, antibody binding to immobilized TSA-protein conjugates was significantly reduced, indicating that many/most antibodies in the sera of these animals recognized the TSA.

2.5 Fusions

Fusions were done on May 14, September 17 and October 30, 1992, using spleen cells from mice #108, #125 and #131, respectively. These fusions were designated F051492, F091792 and F103092. Spleen cells from immunized mice were fused at a ratio of 2:1 with

the P3x63-Ag8.653 cell line. Polyethylene glycol (PEG) was used as the fusion agent. Each donor mouse was boosted 3 days prior to fusion by an intravenous injection of 25 µg of immunogen in saline. Immediately after fusion, hybridomas were plated out in 96-well microtiter plates at 2 X 10^4 spleen cells per well. Each well contained 2 X 10^4 normal peritoneal exudate cells.

A total of 3000 wells were seeded with fused cells, and growth of hybridomas was observed in 2329/3000 wells. Supernatants from 2304 wells were tested in EIAs for binding to TSA-protein conjugate. In these screening assays, 593 supernatants were positive for binding, and 401/593 showed inhibition by free TSA in CIEIAs. Fifty-two hybridomas were chosen for cloning by limiting dilution. From this group, stable clones were derived from 28 hybridomas. Detailed information about the 52 hybridomas which were cloned from these 3 fusions is given in Section 7.0.

2.6 Purification of Monoclonal Antibodies

Each of the 28 stable cloned hybridomas discussed above was injected into Pristane-primed mice for production of ascites. Antibodies were purified from ascites by ammonium sulfate precipitation and affinity chromatography on protein A (Table IV). Antibodies were 90-95% pure as assessed by polyacrylamide gel electrophoresis in the presence of SDS. For most antibodies, the final concentration was adjusted to 20 µM for catalysis testing. A few antibodies were tested at concentrations of 5-10 µM.

2.7 Characterization of Anti-TSA Antibodies

Competitive inhibition EIAs were done to characterize a panel of 20 anti-TSA antibodies with respect to their approximate affinities for TSA, substrate and (in some cases) product. Results of these analyses are shown in Table V. Approximate affinities are indicated by the experimentally measured IC50, or concentration of TSA, substrate or product required to inhibit binding by 50% (this parameter being inversely related to affinity). The panel of anti-TSA antibodies covers a wide range of affinities for TSA. Those antibodies which were found to be catalytic (see below) appear to have very good affinities for TSA, but were not those with the

highest apparent affinities. The subset of catalytic antibodies, indicated in bold type, did have high affinities for the TSA (IC50 0.018 to 0.035 ng/well), modest affinity for substrate (IC50 approximately 3000 ng/well) and low affinity for product (IC50 >>3000 ng/well).

The criteria used to select antibodies for catalysis testing were (1) good binding of TSA, (2) measurable binding of substrate, and (3) little or no binding of product. In retrospect, it appears that the second qualification–measurable binding of substrate–might not have been useful. For the catalytic antibodies derived under this contract, substrate binding was barely measurable in the EIA used to characterize antibodies, and it is possible that adequate binding of substrate by a potentially catalytic antibody would not have been detected. Using only the first and third of the criteria listed above–namely, binding of TSA and no measurable binding of product–199 hybridomas (rather than 52 hybridomas) would have been candidates for cloning by limiting dilution.

2.8 Testing of Antibodies for Catalytic Activity

Twenty-eight anti-TSA antibodies were tested for their ability to enhance the initial velocity of the reaction of potassium cyanide with the enone drug. Product formation was measured by HPLC. Four antibodies were identified which clearly enhanced the reaction rate. Addition of TSA to the reaction mixture in the presence of antibody markedly diminished the reaction velocity (Figure 2), thus indicating the involvement of the antibody binding site in the observed enhancem Preliminary kinetic analysis on one of the antibodies gave values of K_{enone} and K_{KCN} of 51 μM and 9.6 mM, respectively. The value of k_{cat} was 2.33 h^{-1}. The data suggest a rate enhancement of 2×10^4 for the encounter of the enone with antibody cyanide complex, whereas the rate enhancement for encounter of cyanide with the antibody enone complex is 70.

3.0 Detailed Summary of Research Findings After the Midterm of the Contract

3.1 Synthesis of Drug and Transition State Analog (TSA)

3.1.1 Rationale

The objective of the synthetic work was to design and synthesize an α,β-unsaturated ketone which could be used as a drug to react with cyanide under catalysis by an antibody. Not only did the drug have to be synthesized, but a corresponding analog of the transition state for the reaction was also required. Furthermore, this TSA had to be capable of being conjugated to a protein to form an immunogenic material which could stimulate the formation of antibodies capable of stabilizing the transition state for the reaction.

3.1.2 Results

3.1.2.1 Design of the Drug and TSA

Because the reaction of HCN with unsaturated ketones actually involves the cyanide ion as the reactive species, such additions are usually carried out under mildly basic conditions. To determine whether the reaction would occur at physiological pH and temperature, we investigated the uncatalyzed reaction of 4-phenyl-3-buten-2-one with KCN in phosphate-buffered solution at pH 7.4 and 37°C. A second order rate constant of ca. 0.025 M^{-1} hr^{-1} was obtained. Simulations showed that a rate acceleration of about 3×10^6 was needed to have practical protection from cyanide (Cook et al., 1991). This could be achieved by a combination of intrinsic reaction rate acceleration and development of a catalyst. Kinetic modeling results indicate that within the range of affinity constants and catalytic rates associated with known antibodies, pharmacologically relevant concentrations of cyanide and drug could undergo catalytic combination in a time rapid enough to give protection against the toxic effects of cyanide.

Although changing the second order rate constant is a simple way of looking at what is needed to enhance the reaction, the antibody-catalyzed reaction of cyanide with a substrate is more complicated than the chemical reaction. Chart D, adapted from the random sequential bireactant enzyme model (Segel, 1975) describes the equilibrium situation in which both

substrate (enone) and cyanide bind in a stepwise, random fashion to the antibody to form a central complex that releases product. All of the steps are reversible in principle, and the various rate constants involved will have an impact on the rate of the reaction. (In an ordered sequential reaction, only one arm of the diagram would apply.) The product may be released in the form of either enol or ketone. If released as the enol, the product would rapidly equilibrate to predominantly the keto form. The product could also bind to the antibody (product inhibition).

The structures of the drug, product, and associated TSA are shown in Chart C. As noted above, the reaction can be enhanced either catalytically or by increasing the intrinsic rate above that for phenylbutenone. Both approaches are desirable, although care must be taken not to generate a drug which is intrinsically too reactive with nucleophiles. In the drug (compound **C-2** of Chart C), aromatic groups are positioned at the 1 and 2 positions of the propenone chain. Aromatic groups at either of these positions result in an enhancement of the reaction rate (Nagata and Yoshioka, 1977). Electron withdrawing substituents on the 2-phenyl moiety would further enhance the reaction rate, whereas electron donating substituents would reduce it. Thus, modification of the basic structure **C-2** will allow fine tuning of the reaction rate.

The drug molecule terminates in a primary alcohol group. This can be used to introduce various acyl or alcohol groups which could modify the lipophilicity, metabolism or pharmacokinetics of the drug. The alkyl substituent to which the OH is attached forms a link which separates the bulk of molecule from the protein in the hapten-protein conjugate used as the immunogen. In the TSA, the double-bond system mimics the product-like transition state topography, and the presence of an ether oxygen may provide a stimulus for the generation of a hydrogen bond donor site in the antibody, which should assist the reaction.

We postulated that for best catalytic activity, the order of affinity for the antibody should be TSA > drug >> product. The drug must obviously bind to the antibody for catalysis to occur However, the differential in binding energy between the drug and the transition state should

lower the $\Delta H^{\ddagger}$ (the heat of formation for activation). To minimize product inhibition, the product should have as low an affinity for the antibody as possible.

The aryl groups of the TSA, drug and product are likely to be immunodominant and thus have a strong effect on binding affinity. Computer-assisted molecular modeling using the SEARCH and energy minimization force field (Maximin2) features of the SYBYL software (Tripos Associates, St. Louis, MO) identified low energy conformers of the molecules involved (Figure 3). To simplify the modeling, substituents on the aromatic rings were removed, and only the *E*-isomer of the TSA and the enol surrogate of the transition state were studied in detail. The dihedral angles of the planes of the phenyl rings are similar in the enol surrogate for the transition state (45.8°) and the TSA (51.0°). They are also similar in the TSA (51.0°) and the substrate (drug) (53.2°), but differ more between the TSA (51.0°) and the product (74.4°). Thus, product inhibition should be minimized. Both the potential for flexibility in the antibody structure and the availability of numerous other conformations of the small molecules at relatively low energy cost make such modeling studies indicative but not definitive. In the event, some but not all of the antibodies studied had the desired ratio of binding affinities, and the catalytic antibodies fell within this subset.

3.1.2.2 Synthesis of Drug, Product, TSA and Immunogen

Alkylation of the phenolic OH of 4-hydroxyphenyl benzyl ketone with 3-bromopropanol (K_2CO_3, acetone) followed by a Mannich reaction with formaldehyde in the presence of piperidine and acetic acid (cf. Tisselmann and Ribka, 1956), led smoothly to the enone **C-2**. For binding and kinetic studies, **C-2** was purified by preparative HPLC to a single peak on analytical HPLC. Otherwise, crude **C-2** was converted to the cyanoketone **C-3** (KCN, NH_4Cl, N,N-dimethylformamide, water, 100°C.; cf. Agami et al, 1982). Acetylation (Ac_2O, triethylamine, methylene chloride, 4-dimethylaminopyridine) protected the aliphatic hydroxyl group. Treatment of the acetylated cyanoketone with potassium t-butoxide in hexamethylphosphoramide formed an anion that reacted with methyl trifluoromethane sulfonate to give almost exclusively O-alkylation to the desired enol ether (**C-1a**) as an approximately equal mixture of *E*- and *Z*-

isomers (cf. Krow and Michener, 1974). The mixture was carried through subsequent steps. The acetate group was hydrolyzed (K_2CO_3, methanol) and the hydroxyl compound (**C-1b**) converted (carbonyldiimidazole, tetrahydrofuran) to an activated urethane (**C-1c**). All synthetic compounds exhibited ^1H NMR spectra consistent with the assigned structures. Conjugates (**C-1d**) were prepared with molar ratios of 7 and 22:1 from BSA and with molar ratios of 41, 92 and 142:1 from BTg by reaction of the protein with the activated hapten **C-1c** (aqueous $NaHCO_3$, dioxane) and purified by size exclusion chromatography (Bio-Gel P-6). Ratios were determined by differential UV spectrophotometry (Cook et al., 1980) at 265 nm in water.

3.1.2.3 Other Synthetic Work

As described in detail in the midterm report, the synthesis of a 2-napthyl-3-methylcyclohex-2-en-1-one derivative (**E-1**, Chart E) was successfully accomplished. This compound was converted to the 3-cyano-3-methyl-2-napthylcyclohexenone analog and to the corresponding enol TMS ether. The latter compound, although conceivably suitable as a TSA, proved to be too unstable under aqueous conditions to permit conjugation to protein. Unfortunately, neither it nor the corresponding cyclohexenone could be converted to the desired methyl enol ether.

The corresponding enol phosphate from the 3-naphthyl cyanocyclohexanone would provide the desired molecular topography to mimic the cyanide addition transition state, as well as having a polar site for the induction of catalytic sites in the antibody. When diethyl chlorophosphate was added to the reaction mixture of enone and diethyl aluminum cyanide (Chart E), no product was observed. In a model study, TMS vinyl ether **E-2** was treated with fluoride ion and then diethyl chlorophosphate, but no vinyl phosphate was observed. However, when TMS vinyl ether **F-1** (Chart F) was treated with one equivalent of MeLi and then the chlorophosphate, the vinyl phosphate **F-2** was cleanly produced. The vinyl phosphate **F-2** showed good stability toward both conjugating conditions (Me_2NH/0.1M $NaCO_3$) and acetate cleavage conditions ($LiBH_4$). Thus enol phosphate derivatives may be useful as TSAs for Michael-type addition reactons. Unfortunately, the same reaction sequence using MeI to capture the enolate failed to produce the methyl vinyl ether.

A 3-napthylcyclopentenone derivative was prepared from 3-ethoxycyclopent-2-en-1-one by treatment with the napthyl lithio species at -78° to give a crude alcohol which was then exposed to acid (Dowex 50 cation exchange resin) to effect both enol ether cleavage and dehydration to cyclopentenone with a hydroxyalkyloxynaphthyl group in the 3-position. The alcohol was smoothly acylated and the resulting enone was treated with diethyl aluminum cyanide. However, it failed to react, resulting only in recovery of starting material. It also failed to react with KCN and NH_4Cl (cf. Agami et al., 1982).

A vinyl sulfide or vinyl sulfoxide could also be a TSA. Starting with 4'-hydroxychalcone, the β-cyano ketone **G-1** was easily produced by reaction with KCN/NH_4Cl in refluxing DMF/water. A first attempt at synthesizing the vinyl sulfide, made by heating ketone **G-1** with thiophenol in the presence of Montmorilonite KSF clay (Labiad and Villemin, 1989), led to decomposition of starting material.

The β-cyano ketone **G-1** was also treated with thiophenol and gaseous HCl in absolute ethanol (Campaigne et al., 1954), but no vinyl sulfide was observed (reaction 1 of Chart G). The dimethyl ketal **G-2** of ketone **G-1** was easily made using trimethylorthoformate in methanol (reaction 2). However, when this ketal was treated with TMS iodide and hexamethyldisilazane (Miller and McKean, 1982), only the β-cyano ketone **G-1** could be detected (reaction. 3). Attempts were made to react acetylated enone **G-3** with diethylaluminum cyanide with subsequent trapping of the enolate with TMS chloride (Samson and Vanderwalle, 1978). Only the β-cyano ketone **G-4** was observed (reaction 6).

The 3,3-dimethyl analog of the drug **C-1** was also prepared (Chart H). The desoxybenzoin **H-5** was smoothly alkylated with 2-iodopropane (Ozawa et al., 1984) to yield ketone **H-6** in high yield. Ketone **H-6** was then treated with 5% HCl in MeOH, followed by Br_2 in CCl_4, and the crude intermediate was then heated in DMF at 130° with LiCl to produce the β,β-dimethyl enone **H-8**. The crude product was treated with KCN/NH_4Cl (Agami et al., 1982), but no evidence of cyanide addition could be observed. Acetylation of **H-8** yielded enone **H-9**, which reacted with Et_2AlCN only at elevated temperatures to provide very small amounts of the β-

cyano ketone H-10. The unreactive nature of enone H-9 may be attributed to steric crowding at the β-position of the enone, which forces the olefin to twist too far out of the carbonyl plane to allow 1,4 addition.

3.1.2.4 Progress Toward an Immunogen to Induce Catalytic Sites

We also began to investigate other types of TSAs that might facilitate the desired reaction in other and perhaps more efficient ways. In particular, we focused on what has become known as "bait and switch" catalysis (Lerner and Benkovic, 1988; Shokat et al., 1989; Janda et al., 1990). This strategy incorporates a point charge into the hapten with the intent of generating an oppositely charged residue in the binding site of the antibody that would benefit the reaction to be catalyzed.

For the reaction of HCN with an enone, one could envision an advantageously situated carboxylate ion assisting in the deprotonation of HCN in the binding site (Chart I). Since CN^- is the active nucleophile for 1,4-addition to an enone, the shifting of the HCN $\rightleftharpoons$ CN^- + H^+ equilibrium to the right could accelerate the overall reaction to β-cyano ketone.

To take advantage of this rationale, we began to investigate the two types of haptens shown in Chart J. Ketone K-1 (Chart K) was deprotonated with sodium hydride and then treated with freshly distilled β-dimethylaminoethyl chloride in THF. NMR indicated mostly starting material was recovered together with a small amount of what was believed to be O-alkylated (Sperber et al., 1950) product. When the recovered material was treated with acidic conditions to cleave the THP group, only ketone K-2 was obtained in 80% recovery based on starting ketone K-1.

A second possible route to the trimethylammonium hapten J-1 could go through the β-cyano ketone K-3 (Chart K). The cyano group was reduced with Raney-Ni using 50% aqueous hydrazine as the hydrogen source (cf. Adger and Young, 1984). The crude material was then treated with paraformaldehyde (36% in water) and formic acid (95%) at 80°C for 24 h (Pine and Sanchez, 1971). No $-NMe_2$ resonance could be detected with NMR spectroscopy.

In a different approach to the hapten **J-1**, the arylbromide **L-2** was made by alkylation of p-bromophenol with the THP-bromide ether **L-1**. Preparation of the nitrile **L-3** was first attempted as reported in the literature (Sperber et al., 1950; Eisleb, 1941) using sodamide as base (Chart L). Under various reaction conditions, only trace amounts of product could be detected. Finally, benzyl cyanide was first treated with a slight excess of potassium hexamethyldisilazide (KHMDS) and then treated with the alkyl chloride at room temperature for 12 h to provide butyronitrile (**L-3**) in 50% yield after chromatography. The intention was to couple **L-2** and **L-3** via a Grignard reaction, but time did not permit completion of this reaction sequence.

A second type of positively charged hapten is depicted by the N-methyl pyridinium compound **J-2** of Chart J. The synthesis of this type of compound should follow closely that of the previously described imidazolide **C-1c**, starting with a phenacylpyridine (Chart L). The phenacylpyridine **N-5** has been synthesized as shown in Charts M and N. Starting with 4-cyanophenol, the three carbon linker chain was attached as in previous work, using 3-bromo-1-propanol. The crude primary alcohol **M-2** was then protected with a THP group to provide phenylnitrile **M-3** in 73% yield based on starting phenol. α-Picolyllithium reagent was generated by stirring 2-picoline with 2-thienyllithium overnight in a 70:30 solution of benzene and THF (Screttas et al., 1970). Next the nitrile was dissolved in a small amount of benzene and added to the lithiate at room temperature and then heated at 80°C for 2 h (Screttas et al., 1970). After acid work-up and purification by silica chromatography, the phenacylpyridine **M-5** was obtained in 37% yield. It is believed the reason for the modest yield is the sensitivity of the 2-picolyllithium formation step.

The synthesized THP-ether **M-5 (N-1)** was deprotected with aqueous HCl/methanol to provide the primary alcohol (**N-2**) in 79% crude yield (Chart N). The α-methylene ketone (N-3) was formed via a Mannich condensation with formaldehyde (cf. Tiesselmann and Ribka, 1956). This crude enone was carried on without purification. The alcohol group of **N-3** was smoothly acetylated with acetic anhydride yielding the acetoxy enone (**N-4**). The crude enone

was then subjected to KCN (Agami et al., 1982) to yield the β-cyano ketone (**N-5**). These latter reactions were carried out on few milligrams scale as a probe, so no yields are reported here.

During attempts to scale up the above reaction sequence, problems developed with the construction of the phenacylpyridine backbone itself (Chart O). The table in Chart O shows a brief study of the conditions for forming the phenacylpyridine. The first three entries focus on how the generation of 2-picolyllithium affects the product yield. Although the use of n-butyl lithium (n-BuLi) has been cited in the literature as a convenient method (Beumel et al., 1974) for 2-picolyllithium generation, the 2-lithiothiophene method (Screttas et al., 1970) gave the best results (entry 3, Chart O). Further problems were encountered with reproducibility of the attack of lithium reagent on the nitrile as well. We believe the problem may lie with the purity of the 2-lithiothiophene. Time did not permit further study of this or of the conversion of **N-5** to hapten **J-2**.

3.1.3 Experimental Details

Synthesis of 4-(3-Hydroxy-1-propyloxy)phenyl Benzyl Ketone. 4-Hydroxyphenyl benzyl ketone (1 g, 4.72 mmol) and 3-bromo-1-propanol (427 µL, 4.72 mmol) were dissolved in 20 mL of dry acetone with excess K_2CO_3 and refluxed for 8 h. The resulting solids were dissolved in 50 mL $CHCl_3$ and washed with water. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to yield 1.274 g (5.3 mmol, 112% yield) of 4-(3-hydroxy-1-propyloxy)phenyl benzyl ketone as a white solid.

^{1}H NMR ($CDCl_3$): δ 7.98 (d, 2H, ArH); 7.27 (m, 5H, ArH); 6.92 (d, 2H, ArH); 4.23 (s, 2H, C(O)-CH_2Ph), 4.18 (t, 2H, J = 6.03 Hz, -OCH_2-); 3.87 (t, 2H, J = 5.92 Hz, -OCH_2-); 2.06 (q, 2H, J = 5.97 Hz, -CH_2-); 1.57 (s, OH).

Synthesis of 1-[4-(3-Hydroxy-1-propyloxy)phenyl]-2-phenyl-2-propen-1-one (C-2). 4-(3-Hydroxy-1-propyloxy)phenyl benzyl ketone (1.76 g, 5.64 mmol) was dissolved in hot THF (10 mL). Formaldehyde (3 mL, 37%), piperidine (28 µL, 0.28 mmol), and glacial acetic acid (28 mL) were then added and the solution heated at reflux for 20 h. The reaction mixture was poured into water and extracted with $CHCl_3$. The organic phase was dried with $MgSO_4$,

filtered through Celite, and concentrated under vacuum to provide enone **C-2** (1.893 g, 6.7 mmol) in 119% yield. The oily material was suitable for further use without further purification.

^{1}H NMR ($CDCl_3$): δ 7.86 (d, 2H, ArH); 7.3 (m, 5H, ArH); 6.86 (d, 2H, ArH); 5.96 (s, 1H, =C$\underline{H}_2$); 5.51 (s, 1H, =C$\underline{H}_2$); 4.07 (t, 2H, J = 6.18 Hz, -OCH_2-); 3.74 (t, 2H, J = 6.06 Hz, -OCH_2-); 3.4 (br s, 1H, -OH); 1.96 (q, 2H, J = 6.11 Hz, -CH_2-).

Synthesis of 4-[4-(3-Hydroxy-1-propyloxy)phenyl]-4-oxo-3-phenylbutanenitrile (C-3). Enone **C-2** (565 mg, 2 mmol) was dissolved in 80 mL of a DMF:H_2O (9:1) solution containing KCN (260 mg, 4 mmol) and NH_4Cl (160 mg, 3 mmol). The solution was heated at 100°C for 5 h. The reaction mixture was poured into water and extracted with $CHCl_3$. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to give ketone **C-3** as a white solid (454 mg, 1.47 mmol) in 73% yield.

^{1}H NMR ($CDCl_3$): δ 7.9 (d, 2H, ArH); 7.3 (m, 5H, ArH); 6.85 (d, 2H, ArH); 4.80 (dd, 1H, J = 6.7 Hz and 7.9 Hz, C(O)-C$\underline{H}$-Ph); 4.13 (t, 2H, J = 6.06 Hz, -OCH_2-); 3.83 (t, 2H, J = 5.91 Hz, -OCH_2-); 3.07 (dd, 1H, J = 6.6 Hz and 16.8 Hz, -C$\underline{H}_2$CN); 2.86 (dd, 1H, J = 8.0 Hz and 16.8 Hz, -C$\underline{H}_2$CN); 2.05 (q, 2H, J = 6.0 Hz, -CH_2-).

Synthesis of 4-[4-(3-Acetoxy-1-propyloxy)phenyl]-4-oxo-3-phenylbutane-nitrile. The ketone **C-3** (100 mg, 0.32 mmol) was dissolved in 5 mL of dry CH_2Cl_2 with excess acetic anhydride, triethylamine, and a catalytic amount of 4-(N,N-dimethylamino)pyridine (DMAP) and stirred at room temperature for 12 h. The mixture was poured into saturated $NaHCO_3$ and extracted with Et_2O. The organic layer was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to give crude acetate (126 mg). The product was suitable for use without further purification.

^{1}H NMR ($CDCl_3$): δ 7.9 (d, 2H, ArH); 7.3 (m, 5H, ArH); 6.83 (d, 2H, ArH); 4.81 (dd, 1H, J = 6.7 Hz and 7.7 Hz, -C(O)-C$\underline{H}$-Ph); 4.15 (t, 2H, J = 6.25 Hz, -OCH_2-); 3.98 (t, 2H, J = 6.14 Hz, -OCH_2-); 3.05 (dd, 1H, J = 6.6 Hz and 16.8 Hz, -C$\underline{H}_2$CN); 2.85 (dd, 1H, J = 7.8 Hz and 16.8 Hz, -C$\underline{H}_2$CN); 2.1 (m); 2.02 (s, 3H, OAc).

Synthesis of E,Z-4-[4-(3-Hydroxy-1-propyloxy)phenyl]-4-methoxy-3-phenyl-3-butene-1-nitrile (C-1b). The above acetate (353 mg, 1 mmol) was dissolved in 6 mL of hexamethylphosphoramide (HMPA) and cooled to 0°C under a N_2 atmosphere. Potassium t-butoxide (291 mg, 2.6 mmol) was added and stirred at 0°C for 15 min. To this solution methyl trifluoromethane sulfonate (272 µL. 2.4 mmol) was added dropwise via syringe and allowed to react for 15 min. The reaction mixture was poured into water and extracted with $CHCl_3$. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum. The removal of the solvent, HMPA, proved difficult so that the methanolysis of the crude product was carried out by adding 50 mL of methanol and excess K_2CO_3. After 5 h, the reaction mixture was poured into water and extracted with $CHCl_3$. The crude product was worked up as above and purified by SiO_2 column chromatography (3:1 hexane:EtOAc) to yield the methyl vinyl ether **C-1b** in 73% yield (236 mg, 0.73 mmol) starting from the acetoxy ketone. Ether **C-1b** is observed as a mixture of two isomers.

^1H NMR ($CDCl_3$): δ 7.5-6.7 (m, 9H, ArH); 4.19 (t, $-OCH_2-$); 4.05 (t, $-OCH_2-$); 3.87 (two m, $-OCH_2-$); 3.60 (s, $-CH_2CN$); 3.48 (s, $-CH_2CN$); 3.29 (s, -OMe); 3.27 (s, -OMe); 2.1 (m, $-CH_2-$); 2.03 (m, $-CH_2-$).

Synthesis of Imidazolide (C-1c). Methyl vinyl ether **C-1b** (236 mg, 0.73 mmol) was dissolved in 10 mL of dry THF and excess carbonyl diimidazole (CDI) was added. The reaction was stirred for 12 h at room temperature. The THF solution was placed on a plug of SiO_2 and eluted with 1:1 hexane:EtOAc yielding 287 mg (94%) of imidazolide **C-1c**.

^1H NMR ($CDCl_3$): δ 7.5-6.7 (m, 9H, ArH); 4.19 (t, $-OCH_2-$); 4.05 (t, $-OCH_2-$); 3.87 (two m, $-OCH_2-$); 3.60 (s, $-CH_2CN$); 3.48 (s, $-CH_2CN$); 3.29 (s, -OMe); 3.27 (s, -OMe); 2.1 (m, $-CH_2-$); 2.03 (m, $-CH_2-$).

Synthesis of 4-[4-(3-N,N-Dimethylcarbamoyloxy-1-propyloxy)phenyl]-4-oxo-3-phenylbutyronitrile (C-1e). Imidazolide **C-1c** (0.057 mmol crude) was dissolved in 3 mL of THF and excess dimethylamine (40% v/v in H_2O) and excess Et_3N added. The reaction mixture was stirred at room temperature for 12 h. The mixture was then poured into water and

extracted with CH_2Cl_2. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to give 12 mg of crude product. The material was purified on analytical SiO_2 TLC plate (hexane, ethyl acetate) to yield pure carbamate **C-1e** in 21% yield (4.7 mg, 0.012 mmol) from acetate **C-1a**.

^1H NMR ($CDCl_3$): δ 7.88 (d, 2H, ArH); 7.30 (m, 5H, ArH); 7.83 (d, 2H, ArH); 4.80 (m, 1H, C(O)-CH); 4.23 (t, 2H, J = 6.2 Hz, OCH_2-); 4.06 (t, 2H, J = 6.2 Hz, OCH_2-); 3.07 (dd, 1H, J = 6.6 and 16.8 Hz, -CH_2CN); 2.88 (m, 7H, -NMe_2 and -CH_2CN); 2.11 (q, 2H, J = 6.2 Hz, -CH_2-).

Conjugation of Imidazolide (C-1c) with Bovine Serum Albumin (BSA). The BSA (100 mg, ≈0.0015 mmol) was dissolved in 5 mL of 0.1 M $NaHCO_3$ and stirred for 10 min at room temperature. The solution was cooled to 10°C and the imidazolide (8 mg, 0.019 mmol) in 5 mL of 1,4-dioxane was added at once. After 4 h, the reaction mixture was lyophilized. The material formed a milky solution in water, but nevertheless was purified by size exclusion chromatography (Bio-Gel P-6). The conjugate was collected and the samples combined and lyophilized to provide a fluffy white solid. The molar ratio (hapten to protein) was calculated to be 7 by differential UV spectrophotometry at 265 nm in water (Cook et al., 1980).

By the above procedure a second conjugation was performed using 19 mg (0.046 mmol) of imidazolide **C-1c** and 100 mg (0.0015 mmol) of BSA. The molar ratio was calculated as described above to be 22.

Conjugation of Imidazolide C-1c with Bovine Thyroglobulin (BTg). As described above, BTg (50 mg, 7.5 X 10^{-5} mmol) in 2.5 mL 0.1 M $NaHCO_3$ was treated with imidazolide **C-1c** (9.5 mg, 0.023 mmol) in 2.5 mL 1,4-dioxane to yield 39 mg of a white, fluffy solid. The molar ratio was calculated as above to be 41. However, further examination of the product indicated that a filtration step was causing an artifactually low molar ratio result. Therefore, BTg (100 mg, 1.5 X 10^{-4} mmol) in 5 mL 0.1 M $NaHCO_3$ was treated with imidazolide **C-1c** (9.5 mg, 0.023 mmol) in 5 mL 1,4-dioxane to yield 78 mg of a white fluffy solid. The molar ratio was calculated as above to be 92 after centrifugation of a solution of the product. Repetition of the reaction of BTg (50 mg, 7.5 X 10^{-5} mmol) in 2.5 mL 0.1 M $NaHCO_3$ with imidazolide

C-1c (9.5 mg, 0.023 mmol) in 2.5 mL 1,4-dioxane yielded 54 mg of a white, fluffy solid. The molar ratio was calculated as above to be 142.

Synthesis of 3-Cyano-3-methyl-1-trimethylsilyloxycyclohex-1-ene. To a room temperature solution of 3-methylcyclohex-2-en-1-one (20 mg, 0.18 mmol) in 300 µL of dry THF, diethylaluminum cyanide (0.36 mmol, 360 µL of 1 M toluene solution) was added dropwise and allowed to react for 1.5 h. Excess TMSCl (69 µL, 0.54 mmol) and excess pyridine (69 µL, 0.872 mmol) were then added dropwise and the solution stirred for 1.5 h. The mixture was diluted with ether and washed with ice cold saturated NH_4Cl, 5% HCl, and then saturated $NaHCO_3$. The ether layer was dried with Na_2SO_4, filtered through Celite, and concentrated under vacuum to yield the essentially pure TMS vinyl ether.

^1H NMR ($CDCl_3$): δ 4.72 (s, 1H, =CH), 2.5-1.2 (m, ring CH), 1.4 (s, CH_3), 0.2 (s, 9H, $OSi(Me)_3$).

Synthesis of 3-Cyano-3-methyl-1-trimethylsilyloxycyclopent-1-ene (F-1). To a room temperature solution of 3-methylcyclopent-2-en-1-one (48 mg, 0.5 mmol) in 2 mL of dry THF, diethylaluminum cyanide (1.0 mmol, 1 mL of 1 M toluene solution) was added dropwise and allowed to react for 1.5 h. Excess TMSCl (190 µL, 1.5 mmol) and excess pyridine (190 µL, 2.4 mmol) were then added dropwise and the solution stirred for 1.5 h. The mixture was diluted with ether and washed with ice cold NH_4Cl, 5% HCl, and then saturated $NaHCO_3$. The ether layer was dried with Na_2SO_4, filtered through Celite, and concentrated under vacuum to yield the essentially pure TMS vinyl ether.

^1H NMR ($CDCl_3$): δ 4.56 (s, 1H, =CH), 2.5-1.7 (m, ring CH), 1.41 (s, 3H, CH_3), 0.21 (s, 9H, $OSi(Me)_3$).

Synthesis of Diethyl (3-cyano-3-methylcyclopent-1-ene)phosphate(F-2). To a 0°C solution of the above TMS vinyl ether (0.5 mmol crude) in 1 mL of dry THF was added dropwise MeLi (0.5 mmol, 357 µL of 1.4 M ether solution) via syringe. After reaction for 0.5 h, diethyl chlorophosphate (144 µL, 1.0 mmol) was added via syringe and the mixture warmed to room temperature for 1 h. The mixture was then poured into cold saturated $NaHCO_3$ and

extracted with ether. The ether layer was dried with Na_2SO_4, filtered through Celite, and concentrated under vacuum to yield a clear oil.

^1H NMR ($CDCl_3$): δ 5.31 (m, =CH), 4.4-4.0 (m, $P(O)(OCH_2CH_3)_2$), 2.8-2.4 (m, ring CH), 2.1-1.9 (m, ring CH), 1.5-1.2 (m, $-CH_3$ and $P(O)(OCH_2CH_3)_2$).

Synthesis of 3-[6-(3-hydroxy-1-propyloxy)-naphth-2-yl]-cyclopent-2-en-1-one. A solution of 6-bromo-2-[2-(3-oxypropyloxy)tetrahydropyran-2-yl]-napthalene (183 mg, 0.5 mmol) in 5 mL of freshly distilled THF under a N_2 atmosphere was cooled to -78°C, and n-BuLi (1.62 M, 0.5 mmol, 309 µL) was added via syringe. The reaction was stirred at -78°C for 0.5 h and then 3-ethoxycyclopent-2-en-1-one (5) (63 mg, 0.5 mmol in 1 mL of THF) was added via syringe. After 0.5 h at -78°C, the reaction mixture was warmed to room temperature, poured into water and extracted with 2 x 20 mL portions of ether. The ether fractions were dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to produce a light yellow solid. This crude alcohol was dissolved in ~5 mL of MeOH with Dowex-50w cation exchange resin and stirred at room temperature for 3 h. The resin was filtered off and the crude material adsorbed onto SiO_2. The enone was isolated by column chromatography (1:1 H:EA, then 10:1 EtOAc:MeOH) to yield a yellow solid (36 mg, 26% yield).

^1H NMR ($CDCl_3$): δ 8.0-7.1 (m, 6H, ArH). 6.61 (s, 1H, =CH), 4.26 (t, 2H, $-OCH_2-$), 3.92 (5, 2H, $-OCH_2-$), 3.11 (m, 2H, ring CH), 2.60 (m, 2H, ring CH), 2.13 (q, 2H, $-CH_2CH_2CH_2-$).

Synthesis of 3-[6-(3-acetoxypropyloxy)-2-napthyl]-cyclopent-2-en-1-one. The above alcohol (17 mg, 0.06 mmol) was dissolved in 0.5 mL of CH_2Cl_2. Excess acetic anhydride (1 mL), excess Et_3N (1 mL) and a catalytic amount of DMAP were added. The reaction was continued for 12 h. The mixture was then poured into a saturated $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to give essentially a quantitative yield of the acetate. The product was suitable for use without further purification.

^{1}H NMR ($CDCl_3$): δ 8.0-7.1 (m, 6H, ArH), 6.65 (s, 1H, =CH), 4.32 (t, 2H, -OCH_2-), 4.19 (t, 2H, -OCH_2-), 3.15 (m, 2H, ring CH), 2.62 (m, 2H, ring CH), 2.18 (q, 2H, -$CH_2CH_2CH_2$-), 2.08 (s, 3H, OAc).

Synthesis of 2-Phenyl-4-(4-hydroxyphenyl)-4-oxobutyronitrile (G-1). 4'-Hydroxychalcone (224 mg, 1 mmol) was dissolved in DMF-water (9:1) (40 mL) containing KCN (130 mg, 2 mmol) and NH_4Cl (80 mg, 1.5 mmol). The solution was heated at reflux for 4 h, cooled to room temperature, poured into water, and extracted with ether. The ether layer was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to yield a clear oil (116 mg, 46%).

^{1}H NMR ($CDCl_3$): δ 8.1-6.9 (m, 9H, ArH), 4.54 (dd, 1H, J = 6H and 8 Hz, -C(CN)H), 3.65 (dd, 1H, J = 8 Hz and 17.7 Hz, C(O)CH_2), 3.43 (dd, 1H, J = 6 Hz and 17.7 Hz, C(O)CH_2).

Synthesis of 2-Phenyl-4-(4-hydroxyphenyl)-4,4-dimethoxybutanenitrile (G-2). The starting β-cyano ketone **G-1** (100 mg, 0.4 mmol) was dissolved in 3 mL of MeOH with a catalytic amount of p-toluene sulfonic acid. The solution was refluxed for 3 h, diluted with approximately 15 mL of CH_2Cl_2 and extracted from saturated $NaHCO_3$. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated to yield 94 mg (0.32 mmol, 80% yield) of a yellow oil which solidified upon standing at room temperature. The product was suitable for use without further purification.

^{1}H NMR ($CDCl_3$): δ 7.45-6.85 (m, 9H, ArH); 3.40 (dd, 1H, J = 3.5 Hz, 10.2 Hz, CH_2-C<u>H</u>(CN)-Ph); 3.30 (s, 3H, -OMe); 3.05 (s, 3H, -OMe); 2.72 (dd, 1H, J = 10.2 Hz, 14.6 Hz, -CH_2-); 2.18 (dd, 1H, J = 3.5 Hz, 14.6 Hz, -CH_2-).

Synthesis of 4'-(3-Acetoxy-1-propyloxy)chalcone (G-3). 4'-Hydroxychalcone (100 mg, 0.45 mmol) and 3-bromo-1-propanol (90 µL, 1.0 mmol) were dissolved in 2 mL of dry acetone with excess K_2CO_3 and refluxed for 3 h. The solution was decanted and adsorbed onto SiO_2 and purified by SiO_2 column chromatography (1:1 hexane:EtOAc) to give 105 mg (0.37 mmol, 82% yield) of 4'-hydroxypropoxylchalcone.

^{1}H NMR ($CDCl_3$): δ 8.05-6.90 (m, 11H, ArH and -CH=CH-); 4.15 (t, 2H, J = 6.13 Hz, HO-C$\underline{H}_2$-); 3.85 (t, 2H, J = 5.97 Hz, ArO-C$\underline{H}_2$-); 2.71 (s, 1H, -OH); 2.05 (q, 2H, J = 6.01 Hz, -CH_2-).

This alcohol was treated with excess Ac_2O and Et_3N with a catalytic amount of DMAP in CH_2Cl_2 to yield 111 mg (0.34 mmol, 75% yield) of chalcone **G-3** after purification by SiO_2 column chromatography (5:1 hexane:EtOAc).

^{1}H NMR ($CDCl_3$): δ 8.10-6.95 (m, 11H, ArH and -CH=CH-); 4.28 (t, 2H, J = 6.26 Hz, -OC$\underline{H}_2$); 4.12 (t, 2H, J = 6.12 Hz, -OC$\underline{H}_2$-); 2.15 (q, 2H, J = 6.10 Hz, -CH_2-); 2.07 (s, 3H, OAc).

Synthesis of 4-[3-(2-Tetrahydropyranyloxy)-1-propyloxy]phenyl Benzyl Ketone (H-5). Crude 4-(3-hydroxy-1-propyloxy)phenyl benzyl ketone was treated with 3,4-dihydro-2H-pyran (2 g, 24 mmol) in 50 mL of dry CH_2Cl_2 with a catalytic amount of p-toluene sulfonic acid (PTSA) for 3 h. The reaction mixture was then poured into saturated $NaHCO_3$ solution and extracted with $CHCl_3$. Purification by SiO_2 column chromatography gave 1.167 g (3.3 mmol) of the title compound in 70% overall yield.

^{1}H NMR ($CDCl_3$): δ 7.98 (d, 2H, ArH); 7.27 (m, 5H, ArH); 6.93 (d, 2H, ArH); 4.59 (br s, 1H, O-CH-O), 4.22 (s, 2H, C(O)-CH_2-Ph), 4.14 (m, 2H, O-CH_2-); 3.85 (m, 2H); 3.55 (m, 2H); 2.09 (m, 2H, -CH_2-); 1.8-1.4 (m, 6H, ring CH).

Synthesis of 1-[4-[3-(Tetrahydropyranyloxy)-1-propyloxy]phenyl]-2-phenyl-3-methylbutan-1-one (H-6). The above ketone (354 mg, 1 mmol) was dissolved in 2-3 mL of dry THF and the solution cooled to 0°C. NaH (57 mg of 60% oil dispersion, 1.4 mmol) was added to the THF solution and stirred for 10 min. The 2-iodopropane (110 µL, 1.1 mmol) was then added via syringe and the mixture heated to reflux for 5 h. The solution was diluted with 20 mL of Et_2O and washed with water. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to produce 392 mg (0.99 mmol) of ketone **H-6** in 99% crude yield. The product was pure enough for use without further purification.

^{1}H NMR ($CDCl_3$): δ 7.98 (d, 2H, ArH); 7.4-7.1 (m, 5H, ArH); 6.87 (d, 2H, ArH); 4.56 (br s, 1H, O-CH-O); 4.16 (d, 1H, J = 10.1 Hz, C(O)-C$\underline{H}$-); 4.09 (d, 2H, J = 6.3 Hz, O-CH_2-); 3.9-3.7

(m, 2H); 3.6-3.4 (m, 2H); 2.6 (m, 1H, $(CH_3)_2C\underline{H}$-); 2.05 (q, 2H, J = 6.2 Hz, $-CH_2$-); 1.8-1.4 (m, 6H, ring CH); 0.99 (d, 3H, J = 6.4 Hz, $(C\underline{H}_3)_2CH$-); 0.74 (d, 3H, J = 6.7 Hz, $(C\underline{H}_3)_2CH$-).

Synthesis of 1-[4-(3-Hydroxy-1-propyloxy)phenyl]-2-phenyl-3-methyl-2-buten-1-one (H-9). The above ketone (952 mg, 2.4 mmol) was treated with 5% HCl in methanol to provide the primary alcohol which was not purified.

^1H NMR ($CDCl_3$): δ 7.96 (d, 2H, ArH); 7.25 (m, 5H, ArH); 6.89 (d, 2H, ArH); 4.15 (d, 2H, J = 10.3 Hz, C(O)C$\underline{H}$-Ph); 4.07 (t, 2H, J = 6.1 Hz, O-CH_2); 3.77 (t, 2H, J = 5.99 Hz, O-CH_2); 2.6 (m, 2H, $(CH_3)_2C\underline{H}$ and -OH); 1.97 (q, 2H, J = 6.06 Hz, $-CH_2$-); 0.98 (d, 3H, J = 6.4 Hz, $(C\underline{H}_3)_2CH$); 0.73 (d, 3H, J = 6.7 Hz, $(C\underline{H}_3)_2CH$-).

This alcohol was allowed to react with bromine (150 µL, 2.9 mmol) in CCl_4 for 24 h at room temperature. The solvent was removed under vacuum, leaving a dark brown oil which was dissolved in 15 mL of dry DMF. Anhydrous LiCl (excess) was added to the solution which was heated at 130°C for 5 h. The reaction mixture was poured into water and extracted with CH_2Cl_2. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to yield **H-9**.

^1H NMR ($CDCl_3$): δ 7.95 (d, 2H, ArH); 7.4 (m, 5H, ArH); 6.9 (d, 2H, ArH); 4.13 (t, 2H, J = 6.03, $-OCH_2$-); 3.82 (t, 2H, J = 6.0 Hz, $-OCH_2$-); 2.05 (m, 3H, $-CH_2$- and -OH); 1.87 (s, 3H, CH_3); 1.76 (s, 3H, CH_3).

Synthesis of 1-[4-(3-Acetoxy-1-propyloxy)phenyl]-2-phenyl-3-methyl-2-buten-1-one (H-10). The above hydroxy enone (10 mg, 0.032 mmol) was dissolved in 1 mL of dry CH_2Cl_2. Excess acetic anhydride and triethylamine with a catalytic amount of DMAP were added and the solution stirred for 12 h at room temperature. The reaction mixture was poured into Et_2O and washed with a saturated $NaHCO_3$ solution. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum. Purification by SiO_2 column chromatography (8:1 hexane:EtOAc) provided 6 mg (0.017 mmol) of acetate **H-10** in 53% yield.

^1H NMR ($CDCl_3$): δ 7.9 (m, ArH); 7.3 (m, ArH); 6.9 (m, ArH); 4.3 (m, 2H, $-OCH_2$-); 4.1 (m, 2H, $-OCH_2$-); 2.15 (m, 2H, $-CH_2$-); 2.05 (s, 3H, OAc); 1.88 (s, 3H, CH_3); 1.78 (s, 3H, CH_3).

Reduction of ß-Cyano Ketone 9 with Ra-Ni/Hydrazine. The β-cyano ketone **K-3** (100 mg, 0.32 mmol) was dissolved in 3 mL of warm EtOH, and then excess Raney-Ni catalyst (~0.5 mL of a 50% aqueous slurry) was added by pipet. Aqueous hydrazine (2 mL of a 55% solution) was then added slowly by pipet. Evolution of hydrogen gas soon began and continued for 3 h. The crude reaction material was filtered through Celite and then poured into water. The solution was extracted with $CHCl_3$, dried over $MgSO_4$, filtered through Celite, and concentrated under vacuum to give 92 mg of a yellow foam. The ^1H NMR spectrum appeared to represent the reduced material (**K-4**) when compared to the starting nitrile, but the material could not be separated completely from the catalyst to allow for a clear spectrum.

Synthesis of 1-Bromo-4-(3-tetrahydropyranyl-1-propyloxy)benzene (L-2). 3-Bromopropyl-1-tetrahydropyranyl ether **L-1** (2.59 g, 10 mmol crude) was dissolved in acetone with p-bromophenol and excess K_2CO_3, and the solution refluxed for 12 h. The mixture was poured into water and extracted with Et_2O. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum (3.67 g).

^1H NMR ($CDCl_3$): δ 7.40 (m, 2H, ArH); 6.75 (m, 2H, ArH); 4.61 (m, 1H, O-CH-O); 4.05 (t, 2H, J =6.3 Hz, OCH_2); 3.85 (m, -OCH_2); 3.50 (m, -OCH_2-); 2.09 (q, 2H, J =6.3 Hz, -CH_2-); 1.9-1.4 (m, THP ring).

Synthesis of 4-(N-N-dimethylamino)-2-phenylbutyronitrile (L-3). Benzyl cyanide (38 µL, 0.5 mmol) was dissolved in 1 mL of anhydrous toluene under a N_2 atmosphere. Potassium hexamethyldisilazide (1.1 mL, 0.5M in toluene, 0.55 mmol) was added dropwise and stirred at room temperature for 45 min. Freshly distilled 2-dimethylaminoethyl chloride (54 mg, 0.5 mmol) was added and the reaction stirred at room temperature for 12 h. The mixture was poured into water and extracted with CH_2Cl_2. the organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to yield a clear oil (62 mg). The crude material was purified by SiO_2 column chromatography (1:1 hexane:ethyl acetate) to yield the nitrile **L-3** in 50% yield (43 mg, 0.25 mmol).

^{1}H NMR ($CDCl_3$): δ 7.35 (m, 5H, ArH); 4.01 (dd, 1H, J's =8.4 Hz, -CHCN); 2.55-2.40 (m, 1H, $-CH_2-$); 2.40-2.25 (m, 1H, $-CH_2-$); 2.23 (s, 6H, $N(CH_3)_2$); 2.1-1.9 (m, 2H, $-CH_2-$).

Synthesis of 4-(3-Hydroxypropyloxy)benzonitrile (M-2). 4-Cyanophenol (119 mg, 1 mmol) and 3-bromo-1-propanol (181 µL, 2 mmol) were dissolved in 5 mL of dry acetone with excess potassium carbonate and the solution was heated at reflux for 8 h. The reaction mixture was poured into water and extracted with Et_2O. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to give a colorless oil.

^{1}H NMR ($CDCl_3$): 7.56 (d, 2H, ArH); 6.94 (d, 2H, ArH); 4.18 (t, 2H, $-CH_2O$); 3.85 (5, 2H, $-CH_2O$); 2.55 (br s, OH); 2.05 (q, 2H, $-CH_2-$).

Synthesis of 4-(3-(2-tetrahydropyranyloxy)-1-propyloxy)benzonitrile (M-3). The crude alcohol (**M-2**) (1 mmol) was dissolved in 6 mL CH_2Cl_2 with 3,4-dihydro-2H-pyran (455 µL, 5 mmol) and a catalytic amount of p-toluenesulfonic acid (PTSA). The solution was heated at reflux for 5 h, poured into water and extracted with Et_2O. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum. Purification by silica column chromatography gave **M-3** in 73% yield from the starting 4-cyanophenol.

^{1}H NMR ($CDCl_3$): 7.56 (d, 2H, ArH); 6.94 (d, 2H, ArH); 4.58 (br, 1H, O-CH-O); 4.14 (t, 2H, $-CH_2O-$); 3.90 (m, 2H, $-CH_2O-$); 3.50 (m, 2H, $-CH_2O-$); 2.10 (q, 2H, $-CH_2-$); 1.9-1.4 (m, 6H, ring H).

Synthesis of 4-(3-(2-tetrahydropyranyloxy)-1-propyloxy)phenyacyl-2-pyridine (M-5). The THF solvent was evaporated from a 2-thienyllithium solution (420 µL of a 1 M solution) and then replaced with 1 mL of 70:30 benzene:THF solution under a N_2 atmosphere. To this solution 2-picoline (75 µL, 0.76 mmol) was added and the solution stirred overnight at room temperature. Nitrile **M-3** (50 mg, 0.19 mmol) was dissolved in a small amount of benzene and added to the α-picolyllithium solution. The reaction mixture was heated at 80°C for 2 h, cooled to room temperature and then poured into water (1 mL). This solution was acidified with 1 mL of 1 M HCl and allowed to stir for 2 h, then neutralized with excess $NaHCO_3$ solution. The aqueous solution was extracted with $CHCl_3$, dried with $MgSO_4$, filtered through Celite, and

concentrated under vacuum. After purification by silica chromatography (7:1, hexane:ethyl acetate), **M-5** was isolated in 37% yield (25 mg).

^{1}H NMR ($CDCl_3$): δ 8.56 (br d, 1H, pyridine H); 8.03 (d, 2H, ArH); 7.65 (m, 1H, pyridine H); 7.30 (m, 1H, pyridine H); 7.15 (m, 1H, pyridine H); 6.95 (d, 2H, ArH); 4.60 (br s, 1H, O-CH-O); 4.44 (s, 2H, C(O)-CH_2-py); 4.15 (t, 2H, -CH_2O); 3.9 (m, 2H, -CH_2O-); 3.55 (m, 2H, -CH_2O-); 2.10 (q, 2H, -CH_2-); 1.9-1.45 (m, 6H, ring CH).

Synthesis of 1-[4-(3-Hydroxy-1-propyloxy)]-phenacyl-2-pyridine (N-2). The crude THP-ether **M-5** (**N-1**) (25 mg, 0.070 mmol) was dissolved in methanol (5 mL) and 1 M HCl (1.5 mL) and the mixture stirred overnight at room temperature. The reaction mixture was poured into saturated $NaHCO_3$ and extracted with $CHCl_3$. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to yield the primary alcohol (**N-2**) as a light yellow oil in 79% (15 mg, 0.055 mmol) crude yield. The product was suitable for use without further purification.

^{1}H NMR ($CDCl_3$): δ 8.66 (m, 1H, pyridine H); 8.03 (m, 2H, ArH); 7.65 (m, 1H, pyridine H); 7.30 (m, 1H, pyridine H); 7.20 (m, 1H, pyridine H); 7.16 (m, 1H, pyridine H); 6.90 (m, 2H, ArH); 4.44 (s, 2H, C(O)-CH_2); 4.15 (t, 2H, -CH_2O); 3.84 (t, 2H, -CH_2O); 2.04 (m, 2H, -CH_2).

Synthesis of 1-[4-(3-Hydroxy-1-propyloxy)phenyl]-2-(2'-pyridyl)-2-propen-1-one (N-3). 1-[4-(3-Hydroxy-1-propyloxy)]-phenacyl-2-pyridine (**N-2**) (15 mg, 0.055 mmol) was dissolved in 1 mL of THF. Formaldehyde (30 µL, 37% v/v), piperidine (2 µL), and glacial acetic acid (2 mL) were then added and the reaction mixture heated in a sealed tube at 60°C for 17 h. The reaction mixture was poured into water and extracted with CH_2Cl_2. The organic phase was dried with $MgSO_4$, filtered through Celite and concentrated under vacuum. The crude oil was suitable for use without further purification.

^{1}H NMR ($CDCl_3$): δ 8.59 (m, 1H, pyridine H); 7.93 (m, 2H, ArH); 7.65 (m, 1H, pyridine H); 7.45 (m, 1H, pyridine H); 7.20 (m, 1H, ArH); 6.90 (m, 2H, ArH); 6.54 (s, 1H, =CH_2); 5.73 (s, 1H, =CH_2); 4.15 (m, 2H, OCH_2-); 3.85 (m, 2H, CCH_2); 2.08 (m, 2H, -CH_2-).

Synthesis of 1-[4-(3-Acetoxy-1-propyloxy)phenyl]-2-(2'-pyridyl)-2-propen-1-one (N-4). The primary alcohol N-3(assumed 0.055 mmol crude) was dissolved in 1 mL of dry CH_2Cl_2 with excess acetic anhydride, trimethylamine, and a catalytic amount of DMAP and stirred at room temperature for 12 h. The mixture was poured into saturated $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to give crude acetate N-4 (21 mg). The product was suitable for use without further purification.

^1H NMR ($CDCl_3$: δ 8.60 (m, 1H, pyridine H); 7.93 (m, 2H, ArH); 7.65 (m, 1H, pyridine H); 7.45 (m, 1H, pyridine H); 7.21 (m, 1H, pyridine H); 6.88 (m, 2H, ArH); 6.55 (s, 1H, $=CH_2$); 5.73 (s, 1H, $=CH_2$); 4.25 (m, 2H, OCH_2-); 4.10 (m, 2H, OCH_2-); 2.06 (m, 2H, $-CH_2$-); 2.06 (s, 3H, -OAc).

Synthesis of 4-[4-(3-Acetoxy-1-propyloxy)phenyl]-4-oxo-3-(2'-pyridyl)butyronitrile (N-5). Enone (N-4) (crude, assumed 0.055 mmol) was dissolved in 2 mL of a DMF:H_2O (9:1) solution containing KCN (7 mg, 0.11 mmol) and NH_4Cl (5 mg, 0.083 mmol). The solution was heated at 100°C for 5 h. The reaction mixture was poured into water and extracted with $CHCl_3$. The organic phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum.

^1H NMR ($CDCl_3$): δ 8.51 (m, 1H, pyridine H); 7.88 (m, 2H, ArH); 7.60 (m, 1H, pyridine H); 7.23 (m, 1H, pyridine H); 7.13 (m, 1H, pyridine H); 6.78 (m, 2H, ArH); 4.98 (m, 1H, C(O)-CH); 4.15 (m, 2H, OCH_2-); 4.00 (m, 2H, OCH_2-); 3.05 (m, 2H, $-CH_2CN$); 2.00 (m, 2H, $-CH_2$-); 1.97 (s, 3H, OAc).

3.2 Production, Characterization and Purification of Anti-TSA Antibodies

3.2.1 Rationale

The objective of this project was to develop a catalytic antibody-based system for the detoxification of cyanide. The addition of cyanide to a compound of the Michael acceptor type was selected as the detoxification reaction. An enone compound was synthesized for use as the substrate, and a cyanoalkyl enol ether was synthesized as the TSA for the reaction. TSA-

protein conjugates were made for immunization and screening. Spleen cells from immunized mice were used for production of hybridomas in three fusions (F051492, F091792, F103092).

From May 1992 through April 1993 we tested supernatants from 2304 hybridomas for binding to a TSA-protein screening conjugate. In initial screening assays, 593 hybridoma supernatants were positive for binding, and 401/593 showed inhibition by free TSA in CIEIAs. Fifty-two hybridomas were chosen for cloning by limiting dilution. From this group, we derived stable clones from 28 hybridomas.

In order to obtain sufficient quantities of purified antibodies for catalysis testing, each of these 28 hybridomas was injected into Pristane-primed mice for production of ascites. Antibodies were purified from ascites by ammonium sulfate precipitation and affinity chromatography on protein A (Table IV). Antibodies were >90-95% pure as assessed by polyacrylamide gel electrophoresis in the presence of SDS. Final antibody concentrations were adjusted to 5-20 μM for catalysis testing.

Four antibodies--5F4, 5F11, 5G4 and 11F4--increased the initial velocity of the cyanide addition relative to the uncatalyzed reaction (Table VIII). For all four antibodies the increase in reaction rate was inhibited by the presence of excess TSA (Figure 2), indicating that the antibody binding site was involved in enhancement of reaction velocity.

3.2.2 Results

3.2.2.1 Immunization of Mice and Testing of Sera

Three groups of BALB/c mice were immunized with the methoxystilbene TSA coupled to carrier protein. The immunization schedule for groups 1 and 2 is given in Table I, and the schedule for group 3 is given in Table II. Preimmune sera were collected from each mouse before immunizations were begun. Fourteen days after the primary immunization (Bleed 1) and 7 days after the booster injections (Bleed 2 etc.), a small volume of blood was collected from each mouse. Sera were tested in EIA for the presence of antibodies to the binding to immobilized TSA-protein conjugate and in competitive inhibition enzyme immunoassays

(CIEIA) to determine if binding was inhibited in the presence of free TSA. Experimental details are given in Sections 3.2.3.1 and 3.2.3.2.

Results from EIA analyses are shown in Table III for Bleeds 1-2 (groups 1 and 2) and Bleeds 1-4 (group 3). Seven days after the first booster injection (Bleed 2), almost all mice had moderate-to-high levels of serum antibodies. Results from CIEIAs (Figure 1) indicated that many/most antibodies in the sera of these animals recognized the TSA. No binding activity was detected in preimmune sera from these animals (data not shown).

3.2.2.2 Fusion F051492

On May 14, 1992, spleen cells from mouse #108 (immunized with the methoxystilbene-BTg conjugate) were fused at a ratio of 2:1 with the P3x63-Ag8.653 cell line. Polyethylene glycol (PEG) was used as the fusion agent. Three days earlier the donor mouse had been given an intravenous injection of approximately 25 µg of TSA-BTg immunogen. Immediately after fusion, hybridomas were plated out at 2 X 10^4 spleen cells per well in a total of 1200 wells. Each well contained 2 X 10^4 normal peritoneal exudate cells.

Cell growth was observed in 1104/1200 wells (92%). Cells were transferred from 96-well plates to 24-well plates, and supernatants from each culture were tested in CIEIA for binding to the TSA. A total of 346 culture supernatants were identified as binding to the methoxystilbene TSA. These supernatants were tested further in CIEIA for binding to substrate and product, and 25 cell cultures were selected which were secreting antibodies which appeared to have significant levels of reactivity with substrate in addition to TSA.

These 25 hybridoma cell cultures were cloned by limiting dilution. First generation clones (FGC) were tested in CIEIA for binding to TSA, substrate and product. Supernatants from most FGC were analyzed for heavy and light chain isotypes.

FGC from the six most promising hybridomas were injected into Pristane-primed mice for ascites production. The remaining hybridomas were unlikely to have catalytic activity. Twelve hybridomas lost activity (both parent lines and clones). When studied in more detail,

antibodies from four hybridomas were found to recognize TSA only, and four other hybridomas were producing antibodies which bound product to a significant extent.

Antibodies from these six cloned hybridomas were purified partially from ascites fluid by ammonium sulfate precipitation and purified further by affinity chromatography on protein A (Section 3.2.2.6).

Detailed information about the 25 hybridomas from F051492 which were cloned by limiting dilution is given in Section 7.1. Experimental details are given in Section 3.2.3.3.

3.2.2.3 Fusion F091792

On September 17, 1992, spleen cells from mouse #125 immunized with the methoxystilbene-BSA conjugate were fused at a ratio of 2:1 with the P3x63-Ag8.653 cell line using PEG as the fusion agent. Mouse #125 had been boosted 3 days previously by intravenous injection of approximately 25 µg of methoxystilbene-BSA immunogen. Immediately after fusion, hybridomas were plated out in 900 wells at 2 X 10^4 spleen cells per well. Each well contained 2 X10^4 normal peritoneal exudate cells.

Cell growth was observed in 803/900 wells (89%). When colonies covered 5-10% of well bottom, they were transferred to 24-well plates and fed twice before screening. Supernatants from 793 hybridoma cultures were tested in EIA, and 211/793 were positive for binding to TSA.

Supernatants from positive cultures were tested further in CIEIAs for binding to substrate and product. A total of 138 culture supernatants were found to contain antibodies which had measurable affinity to substrate in addition to TSA.

Twenty-five hybridoma cultures from F901792 were cloned by limiting dilution, and stable clones producing anti-TSA antibodies were obtained from 20 hybridomas. Clone supernatants were tested for the presence of antibodies binding to TSA and substrate; supernatants from selected clones were analyzed for heavy and light chain isotypes. All 20 TSA-positive clones were injected into Pristane-primed mice for production of ascites. Antibodies were purified from ascites by ammonium sulfate precipitation followed by affinity chromatography on protein A (Section 3.2.2.6).

Detailed information about the 25 hybridomas cloned by limiting dilution is given in Section 7.2. Experimental details are given in Section 3.2.3.3.

3.2.2.4 Fusion F103092

On October 30, 1992, spleen cells from mouse #131 which had been immunized with the methoxystilbene-BSA conjugate were fused at a ratio of 2:1 with the P3x63-Ag8.653 cell line using PEG as the fusion agent. The donor mouse had been boosted 3 days prior to fusion by intravenous injection of approximately 25 µg of methoxystilbene-BSA immunogen. Immediately after fusion, hybridomas were plated out in 900 wells at 2 X 10^4 spleen cells per well. Each well contained 2 X 10^4 normal peritoneal exudate cells.

Cell growth was observed in 422/900 wells. When colonies covered 5-10% of well bottom, they were transferred to 24-well plates and fed twice before screening. Supernatants from 407 wells were tested for binding to TSA-protein conjugate. Initially, 36/407 were positive for binding, but on retesting in CIEIAs only 10/407 had significant levels of binding and were also inhibited by TSA. Six of 10 were inhibited by TSA only, three were inhibited by TSA and also by substrate, and one was inhibited by TSA and product. One of the three TSA/substrate cultures eventually became negative for binding. Cells from the other two hybridoma cultures were cloned by limiting dilution. Detailed characterization of these 2 hybridoma cultures is given in Section 7.3. Experimental details are given in Section 3.2.3.3.

3.2.2.5 Characterization of Anti-TSA Antibodies

Competitive inhibition EIAs were done to characterize a panel of 20 anti-TSA antibodies with respect to their approximate affinities for TSA, substrate and (in some cases) product. Results of these analyses are shown in Table V. Approximate affinities are indicated by the experimentally measured IC50, or concentration required to inhibit binding by 50% (this parameter being inversely related to affinity). The panel of anti-TSA antibodies covers a wide range of affinities for TSA. Those antibodies which were found to be catalytic (see below) appear to have very good affinities for TSA, but were not those with the highest apparent affinities. The subset of catalytic antibodies, indicated in bold type, did have high affinities for

the TSA (IC50 0.018 to 0.035 ng/well), modest affinity for substrate (IC50 approximately 3000 ng/well) and low affinity for product (IC50 >>3000 ng/well). Experimental details are given in Section 3.2.3.4.

3.2.2.6 Purification of Anti-TSA Antibodies

From the 3 fusions described in Sections 3.2.2.2-4 above, a total of 28 cloned hybridomas were injected into Pristane-primed mice for the production of ascites (Section 3.2.3.5). Each batch of ascites was aliquoted and stored frozen. Each aliquot was analyzed in EIA to determine approximate 50% titers for binding to TSA conjugate. Results are summarized in Table VI.

Antibodies were purified from ascites fluid by ammonium sulfate precipitation followed by affinity chromatography on protein A. In most cases, starting material was 3 mL of ascites. Yields of protein A-adherent material are given in Table IV. Antibodies were 90-95% pure as assessed by polyacrylamide gel electrophoresis in the presence of SDS. For detailed kinetic analyses, an additional batch of antibody 5G4.F11C was purified using 16 mL of ascites fluid as the starting material. Experimental details are given in Section 3.2.3.6 below.

3.2.3 Experimental Details

3.2.3.1 Immunization of Mice

Female BALB/c mice (17-18 g) were obtained from Charles River Laboratories (Raleigh, NC). Each animal was ear-tagged, and a small amount of blood was collected before the immunization procedure was begun.

For the primary immunization, each animal was injected intraperitoneally (ip) with 0.3 mL of an emulsion containing approximately 50 µg of immunogen in bicarbonate (see below), 50 µL of Bacto-Bordetella Pertussis (Difco Laboratories, Detroit, MI), 50 µL of physiological saline and 150 µL of complete Freund's adjuvant. Booster injections (ip) consisted of approximately 25 µg of immunogen in bicarbonate plus 100 µL of physiological saline emulsified with 150 µL of incomplete Freund's adjuvant. Fourteen days after the primary

immunization and 7 days after each booster injection mice were anesthetized with Metofane® and bled from the retro-orbital sinus. Sera were harvested and stored at -20°C.

Stock solutions of both immunogens were prepared in 0.05 M $NaHCO_3$ and stored at -20°C. Methoxystilbene-BTg conjugate (RTI #6990-78; n=142) was weighed out and mixed with 0.05 M $NaHCO_3$ at 1.00 mg/mL. The mixture was sonicated in a bath-type sonicator for 5 min and then clarified by centrifuging in a microcentrifuge for 1 min at 12,000 X g. Methoxystilbene-BSA conjugate (RTI #6990-68.2; n=22) was weighed out and dissolved in 0.05 M $NaHCO_3$ at 1.00 mg/mL.

3.2.3.2 Enzyme Immunoassays (EI) and Competitive Inhibition Enzyme Immunoassays (CIEIA)

Anti-TSA activity in the sera of immunized mice was measured in solid phase EIA as the difference in binding to methoxystilbene-protein screening conjugates and to protein alone. Sera from mice immunized with the methoxystilbene-BTg conjugate RTI #6990-78 were tested using methoxystilbene-BSA screening conjugate RTI #6990-70.1 (n=7), while sera from mice immunized with the methoxystilbene-BSA conjugate RTI #6990-68.2 were tested using the methoxystilbene-BTg screening conjugate RTI #6990-77 (n=41). Bound antibody was detected by the addition of peroxidase-coupled second antibody and substrate. Details of the EIA are given below.

To wells of 96-well flat-bottom microtiter plates was added 50 µL per well of methoxystilbene-protein screening conjugate or protein alone at 0.010 mg/mL in 0.05 M $NaHCO_3$. Plates were sealed and incubated overnight at room temperature. Wells were emptied of plate-coating solution by "flicking" the contents of each plate into a sink or dishpan. To prevent further adsorption of protein, wells were "blocked" by incubation with 300 µL per well of 1% BSA in phosphate buffered saline (PBS) for 30 min at room temperature. Plates were emptied and 25 µL of saline plus 25 µL of antiserum dilution were added per well. Plates were incubated for 2 h at room temperature and washed once with 0.02 M imidazole buffered saline containing 0.02% Tween 20. Peroxidase-coupled second antibody was added at 50 µL per

incubated for 1 h at room temperature. Plates were washed five times with imidazole wash solution, and 100 µL per well of ABTS substrate was added. Absorbance was read at 405 nm.

Competitive inhibition EIAs were performed as described above except that 25 µL of inhibitor solution (2 µg/mL in saline) was added to wells along with antiserum dilution. Inhibitors used were (1) methoxystilbene alcohol RTI #6990-84.2; (2) methoxystilbene acetyl RTI #6990-85.3 and (3) methoxystilbene dimethylurethane RTI #6990-81.2.

Antibody activity in cell culture supernatants, ascites fluid and fractions of purified antibody was tested in solid phase EIAs which were performed as described above. The methoxystilbene-BSA conjugate RTI #6990-70.1 was used as the screening conjugate for samples from F051492. For F091792 and F103092, the methoxystilbene-BTg conjugate RTI #6990-77 (n=41) was used as the screening conjugate until this reagent was used up. After January 12, 1993, the methoxystilbene-BTg conjugate RTI #6990-73 (n=92) was used.

Competitive inhibition immunoassays on hybridoma-derived antibodies were done as described above. For samples from F051492, inhibitors were the TSA acetyl compound RTI #6990-85.3 at 50 ng/well, the enone substrate RTI #6990-52.1 at 1000 ng/well and the product at 50 ng/well. For samples from F091792 and F103092 the TSA alcohol compound RTI #6990-84.2 and the enone substrate RTI #6990-138 and product were used at the same concentrations as for F051492.

3.2.3.3 Fusions

For production of hybridomas secreting antibodies to the methoxystilbene TSA, spleen cells from donor mice were fused with the P3x63-Ag8.653 murine myeloma cell line obtained from the American Type Culture Collection (ATCC). The donor mouse was boosted 3 days before the fusion by intravenous injection of 25 µg of immunogen in saline.

Prior to fusion myeloma cells were maintained in log phase growth at viability >95% in DMEM medium: DMEM (high glucose) containing 20% (v/v) heat-inactivated FBS, L-glutamine (2×10^{-3} M), Na pyruvate (1×10^{-3} M), gentamycin (0.025 mg/mL) and pen-strep (6 µg/mL). After fusion, cells were cultured in HAT medium consisting of DMEM medium plus 1% (v/v)

Nutridoma-NS supplement (Boehringer Mannheim) and HAT (1.361 mg hypoxanthine, 0.0176 mg aminopterin, 0.378 mg thymidine and 2.0 mg BSA fraction V per 100 mL of medium). All cell culture procedures were carried out aseptically using conventional tissue culture techniques.

Spleen cells from the donor mouse were harvested aseptically by perfusion of the spleen with DMEM containing gentamycin (0.025 mg/mL); medium temperature was 37°C. Red blood cells were removed by hypotonic lysis as follows: (1) 4.5 mL sterile H_2O was added to pelleted spleen cells; (2) cell suspension was vortexed gently and incubated for 30 sec; (3) 0.5 mL 10X PBS was added; (4) cell suspension was centrifuged at 274 X g for 10 min; (5) pellet was resuspended in 10 mL of warm DMEM containing gentamycin for cell counts.

For fusion, myeloma cells were mixed with spleen cells at a ratio of 1:2 (myeloma:spleen) and the cell mixture was centrifuged for 5 min at 68 X g (room temperature). Most of the supernatant was removed, and the tube was tapped gently to resuspend the cell pellet in the few drops of residual medium.

PEG-1450 (ATCC; MW 1300-1600) was prepared by melting one vial (2 g) at 56°C, allowing it to cool for 1 min and then adding 3 mL of DMEM containing gentamycin (see above) prewarmed to 37°. The PEG solution was kept at 37°C and used within 2 h.

Warm PEG solution was added to the (loosened) cell pellet during a 1 min period, using 0.7 mL PEG solution per 10^7 spleen cells. After the PEG solution had been added, cells were mixed thoroughly but gently. After 1 min, 2 mL of DMEM containing gentamycin at 37° was added over a period of 2 min and then 8 mL DMEM plus gentamycin over a period of 3 min. The fused cells were centrifuged at 128 X g for 5 min and then resuspended in HAT medium (see above). The cells were plated out by adding 0.1 mL per well to microtiter plates which contained peritoneal exudate cells. These plates had been prepared the day before by adding 2×10^4 PEC in 0.1 mL HAT medium to each well.

3.2.3.4 Characterization of Antibodies

For determination of IC50 values, competitive inhibition immunoassays were done as described in Section 3.2.3.2 above except that serial dilutions of each inhibitor (TSA, substrate, product) were used. TSA was the methoxystilbene alcohol RTI #6990-84.2 and substrate was RTI #6990-52.1. Plates were coated with the methoxystilbene-BTg conjugate RTI #6990-73. Linear regression analysis after logit-log transformation of the data was used to determine the midpoint of each inhibition curve, or the IC50.

Isotype/subisotype analyses were done by EIA on cell culture supernatants using the ImmunoPure Monoclonal Antibody Isotyping Kit I (HRP/ABTS) from Pierce (Rockford, IL) with goat anti-mouse Ig (G+A+M) Coating Antibody.

3.2.3.5 Production of Ascites

BALB/c mice 5-10 weeks of age were injected intraperitoneally (ip) with 0.3-0.5 mL of Pristane (2,6,10,14-tetramethylpentadecane) per mouse. After 7-28 days, mice were injected ip with a suspension of 2 X 10^6 hybridoma cells per mouse in 0.3 mL of cell culture medium. Mice were observed daily after injection of hybridoma cells. When mice showed signs of abdominal swelling (due to accumulation of ascites fluid in the peritoneal cavity), fluid was collected, centrifuged to remove cells and debris and stored frozen.

3.2.3.6 Purification of Anti-TSA Antibodies

Purification of Antibodies 5C10.E4B, 19E2.G5B, 20C10.D11B and 20F2.C2B for catalysis screening. Using 1 mL of ascites as starting material, antibodies were partially purified by precipitation in the presence of 50% ammonium sulfate. Precipitates were resuspended in 1 mL of PBS and dialyzed overnight against 100 mL of PBS. The final volume of each sample was approximately 2 mL.

For affinity chromatography on protein A, 1 mL of each partially purified sample was mixed with 2 mL of ImmunoPure (A) IgG Binding Buffer (Pierce #21001G) and applied to protein A columns (prepacked Protein A AffinityPak columns, Pierce #20356G; vol = 1 mL) which had been equilibrated in binding buffer. When the last of the sample had entered the gel bed,

each column was washed with 15 mL of binding buffer. When the last of the wash/binding buffer had entered the gel bed, adherent protein was eluted using 5 mL of ImmunoPure IgG Elution Buffer (Pierce #21004G). The adherent fraction was neutralized by addition of 0.100 mL of 1.0 M Tris, pH 7.5, per mL of eluate. Protein A columns were regenerated by washing first with 6 mL of 0.1 M citric acid, pH 3.0, and then with 5 mL of water containing 0.02% sodium azide. According to the supplier, each column can be reused a minimum of 10 times without significant loss of activity. Regenerated columns were stored refrigerated.

Fractions obtained from protein A columns were designated as--

"sample": vol = 3 mL; contained approximately 2/3 of the nonadherent material from the original sample;
"wash": vol = 15 mL; contained the remaining nonadherent material from the original sample;
"eluate": vol = 5 mL; contained adherent material from the original sample.

The protein concentrations in these fractions (mg/mL) were estimated by dividing absorbance at 280 nm by 1.4.

Purification of other antibodies for catalysis screening. Using 3 mL of ascites as starting material, antibodies were partially purified by precipitation in the presence of 50% ammonium sulfate. Precipitates were redisolved in 3 mL of binding buffer (see above) and dialyzed overnight against 300 mL of binding buffer. The final volume of each sample was approximately 3 mL.

For affinity chromatography on protein A, 3 mL of each partially purified sample was applied to a protein A "plus" column (prepacked Protein A Plus AffinityPak columns, Pierce #22814; vol = 1 mL) which had been equilibrated in binding buffer. When the last of the sample had entered the gel bed, each column was washed with 15 mL of binding buffer. When the last of the wash/binding buffer had entered the gel bed, adherent protein was eluted using 5 mL of elution buffer (see above). The adherent fraction was neutralized as described above and then dialyzed overnight at 4°C in 500 mL 10 mM Tris, pH 7.5. After dialysis, final sample volumes ranged from 5.5-8.0 mL. Five mL of the dialyzed eluate was concentrated to 20 μM

using Amicon Centriprep 30 concentrators (#4306). Antibody concentrations were obtained by dividing absorbance at 280 nm by 1.4. Protein A columns were regenerated and stored as described above.

Purification of 5G4.F11C for detailed kinetic analyses. Starting material was 16 mL of ascites fluid. The antibody was partially purified by precipitation in the presence of 50% ammonium sulfate. Precipitate was redissolved in 16 mL of binding buffer (see above), and 4 X 4 mL aliquots were dialyzed overnight against 4 X 400 mL of binding buffer. The final volume of partially purified material was approximately 16 mL (4 X 4 mL aliquots).

For affinity chromatography on protein A, each 4 mL aliquot was divided into 2 X 2 mL aliquots, and 2 mL of partially purified material was applied to each of 8 protein A "plus" columns (see above) which had been equilibrated in binding buffer. When the last of the sample had entered the gel bed, each column was washed with 15 mL of binding buffer. When the last of the wash/binding buffer had entered the gel bed, adherent protein was eluted using 5 mL of elution buffer (see above). The adherent fractions were neutralized by addition of 0.100 mL of 1.0 M Tris, pH 7.5, per mL of eluate. The approximately 40 mL of eluate from the 8 columns were dialyzed as 8 X 5 mL aliquots against 10 mM Tris, pH 7.5. Two 5 mL aliquots in separate dialysis bags were dialyzed against 1 liter of Tris buffer. After dialyses, the volumes of aliquots ranged from 6.5-9.5 mL. All aliquots were combined and concentrated to 20 µM using Amicon Centriprep 30 concentrators (#4306).

3.3 Testing of Anti-TSA Antibodies for Catalytic Activity

3.3.1 Rationale

As mentioned previously, Agami et al. (1982) have studied the kinetics of hydrocyanation of enones. These studies were carried out at high pH and temperature and in the presence of high concentrations of organic solvent. They found the rate of 1,4-addition of cyanide to enones to be a second order reaction (first order in both [CN^-] and [enone]). Therefore,

$$dS/dt = -k\,[CN^-][S] \text{ where } S = \text{substrate (enone).} \qquad \text{Eq. 1}$$

The reaction has been shown to depend on the [CN^-] rather than the total cyanide (CN^- + HCN) present in the solution. However, the ratio [CN^-]/([CN^-] + HCN]) will remain constant to a first approximation as long as the pH remains constant. The HCN present at lower pH acts as a reservoir from which CN^- is continuously available.

Under conditions where total cyanide is in large excess throughout the experiments and the pH is constant, the reaction can be treated as a first order kinetic process. The reaction rate is then as follows:

$$dS/dt = -k'[S] \qquad \text{Eq. 2}$$

where the constant k' contains the (constant) cyanide concentration. Half-lives for the reaction can be determined for the total cyanide concentration and pH involved.

For studies of initial reaction velocity $V_{0,\ Bkg}$ for the uncatalyzed reaction, equation 3 applies, where P is the concentration of product

$$V_{0,\ Bkg} = \Delta P/\Delta t = k'([S_0]-P) \qquad \text{Eq. 3}$$

Over the first 5-10% of reaction the rate is expected to be almost linear. If cyanide concentration is essentially constant, a plot of [P] as a function of time t will yield the value of k' as the slope. From this and the cyanide concentration, one can calculate the second order rate constant k_{uncat}. Since we can only estimate the actual concentration of CN^- (from the Henderson-Hassebach relationship), we have chosen to use the total concentration of cyanide (KCN) for rate calculations.

In the case where antibody is present and acting as a catalyst, the situation is more complex. Chart D shows one likely situation. In this case, $V'_{0,Ab}$ can also be determined by assuming linearity over the first 5-10% of the reaction. This can be corrected to $V_{0,Ab}$ by subtracting the background velocity, $V_{0,Bkg}$. Standard reciprocal plots ($1/V_{0,Ab}$) as a function of 1/[enone], at constant [KCN] yield slopes and intercepts which can be replotted versus 1/[KCN] to obtain values of apparent K_m for the substrates, V_{max}, etc. These are used to draw lines to the points of the original reciprocal plots and to determine whether one substrate affects the

binding of the other (Segel, 1975). This process can be repeated for the second substrate and should give similar results.

For screening purposes, it is adequate to simply determine the ratio $V'_{o,Ab}/V_{o,Bkg}$. Ratios significantly greater than 1 indicate enhancement of the rate by the antibody.

Both screening and kinetic characterization require the ability to analyze product quantitatively and preferably at low concentrations in order to characterize the initial velocity. (Since substrate concentrations are changing slowly on a percentage basis at the beginning of the reaction, accurate analysis of this change is relatively difficult; thus product analysis is preferable.) For analysis, we chose HPLC with UV detection, since the compounds exhibit large UV absorbance values. (Unfortunately, it was not possible to simply observe a change in UV absorbance because the ε and λ_{max} values of product and enone were not sufficiently different.) In some early pilot studies, we used an N,N-dimethylurethane derivative of the TSA (**C-1e**) as internal standard for an extraction followed by HPLC. However, for the screening and kinetic studies reported here, it proved more convenient to make direct injections from the reaction mixture onto the HPLC. Since compounds with structures similar to that of the product might act as inhibitors, quantitation was achieved by external standardization.

3.3.2 Results

3.3.2.1 HPLC Analysis

For most analyses a standard curve was prepared each day by duplicate injections of at least three concentrations of product covering the range of anticipated values (generally 0-20 μM product). Peak areas for product were determined in μV-sec and plotted versus μM concentration of product. Table VII shows that good linearity was achieved. Although some daily variation was found, overall the assay was reasonably consistent.

3.3.2.2 Screening of Cell Lines for Catalytic Activity

Background initial velocities were generally determined on the same day as an antibody was screened or the previous day. For the most part, these remained reasonably consistent

[$V_{o,Bkg}$ of 2.370 ± 0.525 (S.D.) μM h^{-1}]. The initial velocities for all 28 antibodies are compared with background velocities in Table VIII.

Four antibodies clearly stand out as having relative velocities of 4 or greater. Some of the other antibodies are apparently accelerating the rate of reaction (e.g., by a factor of 2 for antibody 12B11.C5C), but they were not studied further.

3.3.2.3 Testing for Inhibition by TSA

Each of the four antibodies with a relative velocity of 4 or greater was retested in the presence and absence of the TSA **C-1b**. As shown in Figure 2, the reaction rate was markedly inhibited in the presence of **C-1b**, thus indicating that the antibody binding site was involved in the observed catalysis.

3.3.2.4 Kinetic Analysis of Antibody 5G4

A preliminary kinetic analysis has been carried out on antibody 5G4.F11C. Initial velocities were determined by HPLC analysis of the product over the first 5 to 10% of the reaction for combinations of three concentrations of KCN (2, 5, and 10 mM) and four concentrations of the enone (50, 100, 150 and 200 μM) at pH 7.4. The reaction was shown by regression analysis to be essentially linear over this period. Background velocities were determined and subtracted from the observed velocities in the presence of antibody (20 μM). Figure 4 shows sequential chromatograms of the background reaction for the reaction of a 50 μM concentration of enone with 5 mM KCN. Figure 5 shows the same reaction in the presence of 20 μM antibody 5G4.F11C.

Table IX gives the initial velocities for the various concentrations of enone and KCN studied. Lineweaver-Burk plots of 1/v versus 1/[enone] were made and the resulting slopes and intercepts were replotted versus 1/[KCN] to obtain V_{max} and k_{cat}, together with apparent values of K_{enone}, K_{KCN}, etc. A similar procedure was used in plots of 1/v versus 1/[KCN] and replots of the corresponding slopes and intercepts versus 1/[enone]. The two calculations led to similar results (within 10-20%). Figures 6 and 7 show the data points and the lines resulting from the values obtained from the replots. Since the resulting calculated lines inter-

sect below the line of y = 0, it appears that the two substrates affect each other's binding to the antibody (Segel, 1975). The values of K_{enone} and K_{KCN} were calculated from the intersection values of x to be 51 μM and 9.6 mM, respectively. The value of α (the coefficient for the effect of one substrate on the binding of the other) was calculated to be 2.25 from the interaction of the enone with the antibody-cyanide complex and 2.78 for the interaction of KCN with the antibody-enone complex. The value of k_{cat} was 2.33 hr^{-1}. Based on an average background second order rate constant, k_{uncat}, of 1.18 M^{-1} hr^{-1}, these data indicate a rate enhancement of 2 x 10^4 for the encounter of the enone with the antibody-cyanide complex, whereas the rate enhancement for the encounter of cyanide with the antibody-enone complex is ca. 70. Note that for convenience, all calculations were carried out with the total concentration of KCN, although we do not yet know whether the antibody is interacting with HCN (which is the predominant species at pH 7.4) or with cyanide ion, which is present in only low concentrations. Furthermore, since the pH of tris buffer decreases with increasing temperature, the actual reaction pH was probably below 7.4.

3.3.3 Discussion of Kinetic Analysis

The kinetic data appear to be consistent with a random sequential bireactant enzyme model (Segel, 1975). The steady state equation representing the velocity of this reaction is given below (equation 4) adjusted from Segel (1975).

$$v = \Delta P / \Delta t = \frac{k_{cat}[IgG][enone][KCN]}{\alpha K_{enone}K_{KCN} + \alpha K_{enone}[KCN] + \alpha K_{KCN}[enone] + [enone][KCN]} \qquad \text{Eq. 4}$$

To check on the applicability of this equation, the concentrations of enone and KCN in Table IX were substituted in equation 4 together with the constant values, and the initial velocities were calculated. Figure 8 shows that correspondence between calculated and experimental values. Linear regression analysis of experimental versus calculated velocities gave equation 5.

$$\Delta P/\Delta t \text{ (experimental)} = 1.054\ \Delta P/\Delta t \text{ (calculated)} - 0.288 \qquad \text{Eq 5}$$

The intercept does not differ significantly from zero, and the correlation coefficient $r^2 = 0.9935$.

We therefore used equation 4 to simulate the results from different values of K_{enone}, K_{KCN}, α, k_{cat} and [IgG]. Figure 9 shows two such simulations by numerical analysis of equation 4. We assumed a concentration of KCN = 100 μM, enone = 200 μM and antibody = 50 μM to represent a toxic concentration of KCN in the blood plasma, together with likely achievable concentrations of drug and antibody. The top curve of Figure 9 uses the parameters from the current antibody (5G4.F11C). Over a 30 min interval, this antibody would not usefully reduce cyanide concentrations. However, if the K_{enone} is reduced by a factor of 10 (to 5 μM) and k_{cat} is increased by a factor of 600 (to 23 min^{-1}), the second curve shows a dramatic decline in KCN. A decrease in K_{KCN} by a factor of 10 (to 960 μM) results in the lowest curve, which shows a rapid decrease to nonlethal levels of KCN in less than 5 min.

In section 2.2, it was implied that an uncatalyzed rate of about 1250 $M^{-1}min^{-1}$ (0.025 $M^{-1}h^{-1}$ X 3 X 10^{-6} X 1 h/60 min) would be necessary to achieve a practical reduction in cyanide concentration. Figure 10 shows a computer simulation of such a reaction rate compared with an antibody catalyzed reaction having the parameters above.

3.3.4 Experimental Details

Screening for Catalytic Activity. To a 0.3 mL Reactivial was added 291 μL of antibody solution (pH 7.4) and the system sealed with a Mininert valve cap and taped with Teflon tape. The solution was warmed to 37°C for 0.5 h on a heating block. To this was added 3 μL of 1 M KCN in 1 M Tris buffer (adjusted to pH 7.4) via syringe. The mixture was briefly vortex stirred and equilibrated to 37°C for 0.5 h. The reaction was initiated by adding 6 μL of a 10 mM substrate solution in CH_3CN (total organic solvent 2%) via syringe. The mixture was briefly vortex stirred, and the timer started when the vial was replaced on the heating block. The reaction was followed by HPLC using a Zorbax Rx-C8 reverse phase column with an isocratic mobile phase of 65:35 methanol to water at 0.9 mL/min and monitored at 285 nm. A 20 μL aliquot was withdrawn from the reaction mixture and injected directly onto the column at intervals of 1 min, 0.5 h, 1 h, 1.5 h, and 2 h. Product concentrations were quantified against an external standard curve. The data were analyzed by linear regression using the program JMP (version

2.1, SAS Institute, Cary, NC) to determine the initial velocity, v_o, which was compared to the background v_o. The latter was determined in the same manner using 291 µL of buffer in place of the antibody solution.

Initial Testing for TSA Inhibition. To a 0.3 mL Reactivial was added 281 µL of antibody solution (pH 7.4) in Tris buffer 10 mM) and the system sealed with a Mininert valve cap and taped with teflon tape. The solution was warmed to 37°C for 0.5 h on a heating block. To this was added 3 µL of 1 M KCN in 1 M Tris buffer (pH 7.4) via syringe. The mixture was briefly vortex stirred and equilibrated to 37°C for 0.5 h. Next, 10 µL of 1 mg/mL solution of TSA in methanol was added as above, vortex stirred, and equilibrated to 37°C for 0.5 h. The reaction was initiated by adding 6 µL of 10 mM substrate in CH_3CN (total organic solvent 5%) via syringe, the mixture briefly vortex stirred, and the timer started when the vial was replaced on the heating block. The reaction was followed by HPLC using a Zorbax Rx-C8 reverse phase column with an isocratic mobile phase of 65:35 methanol to water at 0.9 mL/min and monitored at 285 nm. A 20 µL aliquot was withdrawn from the reaction mixture and injected directly onto the column at intervals of 1 min, 0.5 h, 1 h, 1.5 h, and 2 h. Product concentrations were quantified against an external standard curve. The data were analyzed by linear regression using JMP (version 2.1, SAS Institute) to determine the initial velocity, v_o, which was compared to the background v_o. The latter was determined in the same manner.

Lineweaver-Burk Experiments. To a 0.3 mL Reactivial was added 291 µL of antibody solution (pH 7.4) in Tris buffer (10 mM) and the system sealed with a Mininert valve cap and taped with Teflon tape. The solution was warmed to 37°C for 0.5 h on a heating block. To this was added 3 µL of a KCN solution (1, 0.5, or 0.2 M in 1 M Tris, pH 7.4) via syringe. The mixture was briefly vortex stirred and equilibrated to 37°C for 0.5 h. The reaction was initiated by adding 6 µL of a substrate solution (200, 150,100, or 50 µM in CH_3CN, total organic solvent 2%) via syringe. The mixture was briefly vortex stirred, and the timer started when the vial was replaced on the heating block. The reaction was followed by HPLC using a Zorbax Rx-C8 reverse phase column with an isocratic mobile phase of 65:35 methanol to water at 0.9 mL/min

and monitored at 285 nm. A 20 μL aliquot was withdrawn from the reaction mixture and injected directly onto the column at intervals of 1 min, 0.5 h, 1 h, 1.5 h, and 2 h. Product concentrations were quantified against an external standard curve. The data were analyzed by linear regression analysis using JMP (version 2.1, SAS Institute) to determine the initial velocity, v_0, which was compared to the background v_0. The background velocity was determined in the same way using buffer in place of the antibody solution and subtracted from the velocity in the presence of the antibody to give the true v_0 for antibody catalysis.

4.0 Preliminary Toxicity Studies of Enone Substrate (Drug)

4.1 Rationale

Since the long-term goal of this work is to develop a catalytic antibody/drug system which could be used in humans for protection against cyanide exposure, it is important that the components of the proposed treatment system have minimal toxicity in their own right. For this reason we have done preliminary acute toxicity studies in mice with the enone substrate (drug) developed under this contract.

4.2 Results

Preliminary studies were done in mice to assess the acute toxicity of the enone substrate prepared for use with catalytic antibodies. The test compound was dissolved in corn oil and administered orally to mice at doses of 60, 90, 135 and 200 mg/kg. Mice were weighed before dosing, observed intermittently for 24 h after dosing and then reweighed. The corn oil control and the 60, 90 and 135 mg/kg dose groups consisted of two animals each. The high dose group (200 mg/kg) consisted of four animals. Results are shown in Table X.

No overt signs of toxicity were observed in these animals. One of the four high-dose animals died 21-23 h after dosing, and on necropsy was found to have a punctured esophagus. Therefore, the death of this animal was attributed to a technical error in gavaging rather than to the test compound. Control animals lost an average of 1.6% body weight during the 24 h period after dosing. Experimental animals lost an average of 4.5% (200 mg/kg), 0.4% (135 mg/kg), 1.0% (90 mg/kg) and 0.8% (60 mg/kg).

4.3 Experimental

Mice were CD-1 females, 5-6 weeks of age, purchased from Charles River Laboratories (Raleigh, NC). The enone substrate (RTI #6990-138) was weighed out and dissolved at 20 mg/mL in corn oil by sonicating intermittently in a bath sonicator for 15-20 min. The 20 mg/mL solution was used directly for the high dose (200 mg/kg). Dosing solutions at lower concentrations were obtained by 1.5X serial dilutions in corn oil. Animals were weighed and dosed orally by gavage. Dose volumes ranged from 0.20-0.27 mL.

5.0 Conclusions and Recommendations

Work carried out under this contract has demonstrated the feasibility of developing antibodies which catalyze the reaction of cyanide with an α,β-unsaturated ketone substrate. This is a significant development in that it represents one of a relatively small number of reports of antibody catalyzed C-C bond formation, in this case via a Michael addition reaction. The methoxystilbene TSA designed and synthesized for this work apparently mimics the actual transition state for the reaction, and the enone substrate works well and is relatively nontoxic in mice.

One of the catalytic antibodies produced under this contract (5G4) has been analyzed to determine kinetic parameters. Results indicate that this antibody catalyzes a random sequential bimolecular reaction between cyanide and enone with kinetic parameters of $K_{m,enone}$, $K_{m,KCN}$, and k_{cat} of 50 μM, 9.6 mM and 2.33 h^{-1}, respectively.

Based on the treatment of Segel (1975) for random sequential bimolecular reactions, we have estimated the values for $K_{m,enone}$, $K_{m,KCN}$, and k_{cat} which would be required for removal of 100 μM cyanide (as KCN) to minimal levels in 5 min. Assuming a drug concentration of 200 μM and an antibody concentration of 50 μM, the following improvements in kinetic parameters would be required relative to catalytic antibody 5G4:

Reduce K_m for both reactants by 10X;
Increase k_{cat} by 600X.

The necessary values for K_m and k_{cat} appear to be attainable ones for catalytic antibodies. (Different combinations of changes in the constants could give the same result.)

There are two approaches to attaining these goals in a catalytic antibody/cyanide detoxification system. One approach is *de novo* production of additional catalytic antibodies using TSAs modified by (1) addition of charged groups as described by Janda et al. (1990) (bait and switch approach) or (2) slightly increased separation of "subsites" in the TSA in order to reduce interference between corresponding subsites for binding of reactants in the antibody active

site. A second approach is modification of an existing catalytic antibody such as 5G4 by either directed or random mutagenesis.

In the first approach, modified TSAs would be used as haptens for the production of additional monoclonal antibodies by conventional hybridoma technology. Anti-TSA antibodies would be tested for catalytic activity and characterized as was done under the current contract. This approach is straightforward from the hybridoma/antibody standpoint. Considerable progress in the design and synthesis of a modified TSA has been made during the current contract. Given the degree of success attained using the methoxystilbene TSA, it is reasonable to use this structure as a basis for further work.

Improvement of existing catalytic antibodies by protein engineering techniques is a potentially powerful approach to developing antibodies for specific applications. Until recently the primary focus of catalytic antibody work has been on demonstrating the versatility of antibodies as catalysts for different kinds of reactions rather than on improving the properties of existing antibodies. As a result, relatively little attention has been given as yet to significantly improving the properties of existing catalytic antibodies. To our knowledge, there are only two reports describing mutagenesis of catalytic antibodies. Baldwin and Schultz (1989) reported an increase in k_{cat} by 45X, but this was accompanied by a decrease in substrate binding by 2X-8X. Jackson et al. (1991) reported an 8X increase in k_{cat} and a 2X increase in K_m. Improvements of this magnitude would not be adequate for in vivo applications of antibody 5G4, but it seems unlikely that these two reports indicate the limits of what can be done using the site-directed mutagenesis approach. For example, there are numerous reports of increases in binding affinity of antibodies by site-directed mutagensis and by chain shuffling, and large increases on the order of 200-300X have been reported by Sharon (1990), Bedzyk and Voss (1991) and Marks et al. (1992).

A combination of the two approaches mentioned above would give a high probability of success in realizing the eventual goal of this work--namely, the development of a drug/catalytic antibody system which can be used in humans for protection against cyanide exposure. It is

unlikely that antibody 5G4 *per se* can be modified sufficiently by genetic engineering to achieve the necessary level of catalytic activity. [We do not know whether this is true of the other 3 catalytic antibodies produced under this contract.] However, modifications of the TSA to produce additional catalytic antibodies which will in turn be fine-tuned by genetic engineering would be a viable and practical approach to this goal.

6.0 Characterization of Hybridomas Cloned by Limiting Dilution

6.1 Hybridomas from F051492

2B7. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17; cell growth was found in 30/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 12/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). First generation clones (FGC) were tested for binding on June 18, and 0/42 were positive. The parent line tested negative for binding on July 1. No further work was done with this cell line.

2B8. This hybridoma was cloned by limiting dilution on June 18, 1992. Clone plates were examined on June 29; cell growth was found in 3/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 0/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 30, and 0/3 were positive for binding. The parent line tested negative for binding on July 1. No further work was done with this cell line.

2D3. This hybridoma was cloned by limiting dilution on June 18, 1992. Clone plates were examined on June 29; cell growth was found in 17/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 12/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 30, and 0/29 were positive for binding. The parent line tested negative for binding on July 1. No further work was done with this cell line.

2G5. This hybridoma was cloned by limiting dilution on June 18, 1992. Clone plates were examined on June 29; cell growth was found in 6/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 3/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 30, and 9/9 were positive for binding. The parent line was positive for binding and inhibition on July 7, with inhibition by TSA (50 ng/well) > substrate (1000 ng/well) > product (50 ng/well). Three FGC were tested for binding and inhibition on July 9; all were inhibited by TSA, but inhibition by substrate (1000 ng/well)

was approximately equal to inhibition by product (50 ng/well). [The remaining FGC were tested on July 14 with the same result.] Since the inhibition profile of the FGC did not match that of the parent line, the parent line was recloned by limiting dilution on July 10. Eleven FGCs were isotyped on August 7, 1992; all were positive for γ_1 and κ.

The second set of cloning plates were examined on July 21. Cell growth was found in 11/60 wells on the B plate and 4/60 wells on the C plate. Thirteen of 15 FGC were positive for binding on July 22. Eleven FGC were tested for binding and inhibition on August 4-5. The inhibition profile was that product (50 ng/well) was either equal to or slightly greater than substrate (1000 ng/well). On July 23, the parent line was found to be better inhibited by product (50 ng/well) than by substrate (1000 ng/well).

Since we were unable to obtain stable clones with reasonable affinities for substrate, we did no further work on this cell line.

5B5. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. Cell growth was found in 35/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 29/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 18, and 4/64 were positive for binding. The 4 FGC were tested for binding and inhibition on June 25-26. One clone was negative, and 3 clones had inhibition by substrate (1000 ng/well) approximately equal to inhibition by product (50 ng/well). The parent line was retested on July 1 and found to be inhibited by substrate but not by product. Since the inhibition profile of the FGC did not match that of the parent line, the latter was recloned by limiting dilution on July 2.

The second set of clone plates were examined on July 13. Cell growth was observed in 1/60 wells on the B plate and in 1/60 wells on the C plate. Both of these FGC were negative for binding on July 15. The parent line tested negative for binding on July 23. No further work was done with this cell line.

5C10. This hybridoma was cloned by limiting dilution on June 18, 1992. Clone plates were examined on June 29. Cell growth was found in 26/60 wells on the B plate (plated out at

an average concentration of 1.0 cells/well) and 13/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 30, and 33/39 were positive for binding. Thirty-two FGC were tested for binding and inhibition on July 7-8 with the result that inhibition by substrate (1000 ng/well) was slightly greater than inhibition by product (50 ng/well). A similar result had been obtained for the parent line on July 1. Supernatants from the 32 positive FGC were isotyped on July 8. All were positive for γ1 and κ. The FGC 5C10.E4B was injected into Pristane-primed mice for production of ascites on July 15. Ten lots of ascites (approximately 1 mL each) were frozen down between July 29 and August 7 and titered on September 30. Seven had approximate 50% titers of 50,000-100,000; three were negative for binding. Antibody from 5C10.E4B was partially purified by ammonium sulfate precipitation on October 2 and further purified by affinity chromatography on protein A on October 11.

5D10. This hybridoma was cloned by limiting dilution on June 18, 1992. Clone plates were examined on June 29. Cell growth was found in 27/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 17/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 30, and 0/44 were positive for binding. The parent line tested negative for binding on July 1. No further work was done with this cell line.

6B4. This hybridoma was cloned by limiting dilution on July 23, 1992. Clone plates were examined on August 4. Cell growth was found in 5/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 3/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on August 5, and 0/8 were positive for binding. The parent line tested negative for binding on August 5. No further work was done with this cell line.

7E8. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. Cell growth was found in 2/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 0/60 wells on the C plate (plated out at an average

concentration of 0.5 cells/well). FGCs were tested on June 18, and 2/2 were positive for binding. The 2 FGC were tested for binding and inhibition on June 26. The inhibition profile was TSA (50 ng/well) > substrate (1000 ng/well) > product (50 ng/well). Inhibition by substrate and product was not very striking. Isotype analysis of supernatants from these 2 FGC on June 29 indicated the presence of $\gamma 1$, κ and λ chains and the possible presence of $\gamma 3$ chains. Therefore the 7E8.E9B FGC was subcloned on July 2. There was no cell growth in the subcloning plates. The 7E8.E9B clone tested negative for binding on July 16. No further work was done with this cell line.

8E9. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. Cell growth was found in 4/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 0/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 18, and 1/4 was positive for binding. This FGC did not grow in culture. The parent line was tested for binding and inhibition on July 1 and showed inhibition by substrate as well as by TSA. The parent line was recloned by limiting dilution on July 2. The second set of clone plates was examined on July 13. Cell growth was obtained in 10/60 wells on the B plate and 2/60 wells on the C plate. Twelve of twelve FGC were negative for binding on July 15. The parent line tested negative for binding on July 23, and no further work was done with this cell line.

8F5. This hybridoma was cloned by limiting dilution on June 18, 1992. Clone plates were examined on June 29; cell growth was found in 25/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 12/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 30, and 32/37 were positive for binding. The parent line was tested for binding and inhibition on July 1 and showed inhibition by TSA only. Twenty-nine FGC were retested for binding and inhibition on July 7. Three were negative for binding, and for the others inhibition by substrate (1000 ng/well) was approximately equal to inhibition by product (50 ng/well). Five FGC were tested on July 9; one was negative and four were inhibited by TSA only. Isotype analysis of supernatants from 8

FGC indicated the presence of κ heavy chains and light chains. The 8F5.E3C clone was injected into Pristane-primed mice for production of ascites on July 15. Eight lots of ascites were frozen down between July 29-31; all were negative for activity when tested on September 30.

8G8. This hybridoma was cloned by limiting dilution on June 18, 1992. Clone plates were examined on June 29; cell growth was found in 19/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 12/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 30, and 0/31 were positive for binding. The parent line tested negative for binding on July 1. No further work was done with this cell line.

9E3. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. Cell growth was found in 0/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 1/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). The FGC tested positive for binding on June 18. When tested for binding and inhibition on June 25, the 9E3.G7C clone showed significant inhibition by product. Therefore, no further work has been done with this cell line.

10D6 This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. Cell growth was found in 6/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 6/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 18, and 10/11 were positive for binding. A total of 9 FGC were tested for binding and inhibition on June 26 & 30 and July 1. All showed inhibition by TSA (50 ng/well) > substrate (1000 ng/well) > product (50 ng/well). Three FGC and the parent line were retested for binding and inhibition on July 7-8 with the same result. Supernatants from 8 FGC were isotyped and were positive for γ2a and κ questionable for λ. The 10D6.B3C clone was subcloned on July 10. Subcloning plates were examined on July 21 with the result that cell growth was found in 14/60 wells on the B plate and 6/60 wells on the C plate. All 20 second generation clones (SGC) were positive for

binding (July 22). Seventeen SGC were tested for binding and inhibition on August 4-5, and all showed inhibition by TSA and substrate with relatively little inhibition by product. The 10D6.B3C FGC was injected into Pristane-primed mice for production of ascites on July 15. Seventeen SGCs were isotyped on August 7 with the result that all were positive for γ_{2a} and κ. Six lots of ascites (approximately 1 mL each) from FGC 10D6.B3C were frozen away between July 29-31. Approximate 50% titers of FGC ascites (tested September 30) were 50,000–200,000. The SGC 10D6.B3C.D4C was injected for ascites on August 17; 11 lots of ascites (approximately 1 mL each) were frozen away between August 29 and September 3. Most had 50% titers >50,000. Antibody from 10D6.B3C.D4C was partially purified from ascites by ammonium sulfate precipitation on October 2 and further purified by affinity chromatography on protein A on October 26.

11D5. This hybridoma was cloned by limiting dilution on July 23, 1992. Clone plates were examined on August 4. Cell growth was found in 5/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 2/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on August 5, and 0/7 were positive for binding. The parent line also tested negative for binding on August 5. No further work was done with this cell line.

12G8. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. No cell growth was found. Since the parent line had become negative for binding, no further work was done with this cell line.

14E9. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. Cell growth was found in 28/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 13/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 18, and 34/34 were positive for binding. Twenty-two FGC were tested for binding and inhibition on July 25 & 30 and showed the inhibition by TSA substrate and product. Inhibition by substrate (1000 ng/well) exceeded inhibition by product (50 ng/well). The parent line tested on July 1 showed the same

inhibition profile. Supernatants from 19 FGC were isotyped on June 29 and July 8. Nine clones were positive for γ2b and κ; 8 were positive for γ1 as well as for γ2b and κ; and 2 were positive for μ, γ1 and λ as well as γ2b and κ. The 14E9.D2B FGC was tested for binding and inhibition on July 16, and this FGC plus the 14E9.E10B (γ2b, κ) were tested on July 23. In both assays the inhibition profile was TSA (50 ng/well) > substrate (1000 ng/well) > product (50 ng/well). The 14E9.D2B FGC was injected into Pristane-primed mice for production of ascites on July 15. Since the isotyping results had been bizarre for several FGC, the 14E9.D2B (γ2b, κ) FGC was subcloned on July 2, but no SGC were obtained. This clone was subcloned again on July 23, and subcloning plates were examined on August 4. Cell growth was observed in 15/60 wells on the B plate and in 12/60 wells on the C plate. Twenty-five of twenty-seven SGC tested positive for binding on August 5. Twenty-seven SGC were tested in a competitive inhibition immunoassay for binding and inhibition on August 12. Twenty-three showed inhibition by TSA (50 ng/well) and substrate (1000 ng/well); product inhibition was also observed (50 ng/well). Twenty-four SGC were isotyped on August 13 with the result that 22 were positive for γ_{2b} and κ, one was positive for γ_{2b}, γ_{2a}, and κ, and one was positive for γ_{2b}, κ, and λ. The SGC 14E9.D2B.E8B was injected for ascites on August 17. Aliquots of ascites (approximately 1 mL each) from FGC 14E9.D2B were frozen July 28-29; six had titers of 50,000-100,000 and one had a titer of 10,000-50,000 (tested September 30). From SGC 14E9.D2B.E8B nine lots of ascites (approximately 1 mL each) were frozen down between August 28 and September 6. Most of these had titers of 10,000-50,000 (tested September 30). Antibody was partially purified by ammonium sulfate precipitation from 14E9.D2B ascites on October 2 and further purified by protein A affinity chromatography on October 26.

14G2. This hybridoma was cloned by limiting dilution on June 18, 1992. Clone plates were examined on June 29, cell growth was found in 15/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 6/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 30, and 0/21 were positive

for binding. The parent line tested negative for binding on July 1. No further work was done with this cell line.

15B11. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. Cell growth was found in 11/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 7/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 18, and 6/16 were positive for binding. Two FGC were tested for binding and inhibition on June 26 & 30 with the result that very little inhibition was observed with substrate and product. Retesting the parent line on July 1 also suggested that 15B11 might be a TSA only, and it was decided not to make ascites from this cell line. Supernatants from 2 FGC were isotyped as $\gamma 1$ and κ on June 29 and July 8.

16C5. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. Cell growth was found in 0/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 1/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). This FGC tested positive for binding on June 18, but did not grow in tissue culture. When the parent line was retested for binding and inhibition on July 7, inhibition by substrate @ 1000 ng/well was approximately equal to inhibition by product @ 50 ng/well. Therefore we decided not to do further work with this cell line.

16D6. This hybridoma was cloned by limiting dilution on June 18, 1992. Clone plates were examined on June 29; cell growth was found in 29/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 17/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 30, and 0/46 were positive for binding. The parent line tested negative for binding on July 1. No further work was done with this cell line.

17C6. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. No clones were obtained, and the parent line became negative for binding. Therefore no further work was done with this cell line.

19E2. This hybridoma was cloned by limiting dilution on July 2, 1992. Clone plates were examined on July 13. Cell growth was found in 4/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 0/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on July 15, and 4/4 were positive for binding. The FGC were tested for binding and inhibition on July 23 and August 4-5 with the result that binding was observed with TSA only. A similar result was obtained with the parent cell line on July 16, and no further work was done with this cell line. Six FGC were isotyped on August 7 with the result that all were positive for γ_3 and λ (2 were $\pm$ for κ). The FGC 19E2.G5B was injected for ascites on August 17, and five lots of ascites (three approximately 0.5 mL and two approximately 1 mL) were frozen between September 1-6. Approximate 50% titers were 10,000-50,000 (September 30). Antibody was partially purified from 19E2.G5B ascites by ammonium sulfate precipitation on October 2 and further purified by affinity chromatography on protein A on November 12.

20C10. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. Cell growth was found in 25/60 wells on the B plate (plated out at an average concentration of 1.0 cells/well) and 9/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 18, and 30/32 were positive for binding. The parent line tested positive for binding and inhibition on July 7 with the inhibition profile TSA (50 ng/well) > substrate (1000 ng/well) > product (50 ng/well). Supernatants from 11 FGC were isotyped on June 29 with the result that 10 were positive for 1 and while 1 was positive for γ1, γ2b and κ. The 20C10.D11B FGC was injected into Pristane-primed mice on July 15 for production of ascites. Six lots of ascites (approximately 1 mL each) were frozen on July 26. Approximate 50% titers were 50,000-100,000 (September 30). Antibody from 20C10.D11B ascites was partially purified by ammonium sulfate precipitation on October 2 and further purified by affinity chromatography on protein A on November 12.

20F2. This hybridoma was cloned by limiting dilution on June 8, 1992. Clone plates were examined on June 17. Cell growth was found in 28/60 wells on the B plate (plated out at

an average concentration of 1.0 cells/well) and 28/60 wells on the C plate (plated out at an average concentration of 0.5 cells/well). FGCs were tested on June 18, and 54/54 were positive for binding. Forty-four FGC were tested for binding and inhibition on June 25 with the result that inhibition by TSA (50 ng/well) > substrate (1000 ng/well) > product (50 ng/well). Supernatants from 41 FGC were isotyped on June 29 with the following results: 12 were positive for γ1 and κ; 25 were positive for γ1 and κ and questionable for λ; 1 was positive for γ1, γ 2b and κ; 2 were positive for γ1, γ2b and κ and questionable for λ; 1 was positive for γ1, γ2a and κ. The 20F2.C10B FGC was injected into Pristane-primed mice on July 15 for production of ascites. Eight lots of ascites (approximately 1 mL each) were frozen between July 28-31. Most showed approximate 50% titers of 10,000-50,000 (September 30). Antibody from 20F2.C2B ascites was partially purified by ammonium sulfate precipitation on October 2 and further purified by affinity chromatography on protein A on November 12.

6.2 Hybridomas from F091792

1F4. This hybridoma was cloned by limiting dilution on October 9, 1992. Clone plates were examined on October 19; cell growth was found in 7/60 wells on the B plate and 7/60 wells on the C plate. FGCs were tested for binding on October 20, and 11/12 were positive. Three FGC were tested in competitive inhibition immunoassays on October 27 and 30 with the result that inhibition was observed with TSA (50 ng/well) and substrate (1000 ng/well), while no product inhibition (50 ng/well) was observed under the conditions used. Supernatants from two FGC were isotyped on October 30; both were positive for γ_1 and κ. Since the activity profile of this antibody looked promising and because cell growth was relatively poor, the parent line was recloned by limiting dilution on November 6. [The parent line was positive for binding when tested on November 3.] Cloning plates were examined on November 17; cell growth was found in 46/60 wells on the B plate (plated out at an average concentration of 1.0 cell/well) and in 33/60 wells on the C plate (plated out at an average concentration of 0.5 cell/well). FGCs were tested for binding on November 18 with the result that two were negative and all others were positive. A total of 62 FGC were tested in competitive inhibition

immunoassays (November 24 and December 1). Binding was inhibited by TSA at 50 ng/well and to a lesser extent, by substrate at 1000 ng/well. No inhibition was observed with product at 50 ng/well. Ten FGC were isotyped as positive for γ_1 and κ on November 25. Clone 1F4.C11C was injected into Pristane-primed mice for production of ascites on December 18. Ascites fluid was collected between December 30 and January 11. Approximately 13 mL of ascites fluid was collected, aliquot and stored frozen. The approximate 50% titer of the ascites was 50,000-100,000. Antibody 1F4.C11C was partially purified by ammonium sulfate precipitation on January 23 and further purified by affinity chromatography on protein A on January 28. Starting material was 3 mL of ascites.

1G9. This hybridoma was cloned by limiting dilution on October 22, 1992. Clone plates were examined on November 3. Cell growth was found in 23/60 wells on the B plate and in 14/60 wells on the C plate. FGCs were tested for binding on November 4 and 1/37 was positive. Supernatant from the parent hybridoma was positive for binding on November 3, and the parent line was recloned by limiting dilution on November 6. Cloning plates were examined on November 17; cell growth was found in 25/60 wells on the B plate and in 19/60 wells on the C plate. All 41 FGC were negative for binding on November 18. Since the parent line tested positive for binding on November 17, this hybridoma was cloned by limiting dilution for the third time on November 19. Clone plates were examined on November 30, and cell growth was observed in 20/60 wells on the B plate and in 15/60 wells on the C plate. One of the 35 FGC tested positive for binding on December 1. This clone (1G9.F3B) was isotyped as positive for γ_1 and κ on December 9. The clone was tested in a competitive inhibition immunoassay on December 8 with the result that binding was inhibited by TSA at 50 ng/well and slightly inhibited by substrate at 1000 ng/well. No inhibition was observed with product at 50 ng/well. Clone 1G9.F3B was injected into Pristane-primed mice for production of ascites on December 18. Approximately 11.5 mL of ascites fluid was collected between December 31 and January 14. Ascites was aliquoted and stored frozen. Approximate 50% titers were 100,000-200,000. Monoclonal antibody 1G9.F3B was partially purified from 3 mL ascites by

ammonium sulfate precipitation (January 23) and by affinity chromatography on protein A (January 28).

3E7. This hybridoma was cloned by limiting dilution on October 22, 1992. Clone plates were examined on November 3. Cell growth was found in 11/60 wells on the B plate and in 2/60 wells on the C plate. None of the FGCs tested positive for binding on November 4. The parent cell line was recloned by limiting dilution on November 6. Clone plates were examined on November 17 with the result that cell growth was observed in 33/60 wells on the B plate and in 22/60 wells on the C plate. All 55 FGC tested negative for binding on November 18. Since the parent line tested negative on November 16, no further work was done with this cell line.

3E10. This hybridoma was cloned by limiting dilution on October 21, 1992. Clone plates were examined on November 2. Cell growth was found in 7/60 wells on the B plate and in 5/60 wells on the C plate. None of the FGCs tested positive for binding on November 3, although the parent line was still positive. The parent hybridoma was recloned by limiting dilution on November 5. Cloning plates were examined on November 16 with the result that cell growth was found in 8/60 wells on both the B and the C plates. All 16 FGC tested negative for binding on November 17. Since the parent line was also negative on November 17, no further work was done with this cell line.

4C5. This hybridoma was cloned by limiting dilution on October 22, 1992. Clone plates were examined on November 3, and cell growth was found in 7/60 wells on the B plate and in 5/60 wells on the C plate. Ten of twelve FGCs tested positive for binding on November 4. Three FGC were tested in competitive inhibition immunoassays on November 10 and 12. Inhibition was observed with TSA (50 ng/well), but significant inhibition was not observed with substrate (1000 ng/well) or product (50 ng/well). The competitive inhibition assays were done [illegible] antibody excess so the significance of these results is not clear. Six FGC were isotyped on November 12. Five were positive for γ_1 and κ; one was positive for γ_1 and γ_{2a} and for κ. Clone 4C5 D10C was injected into Pristane-primed mice for production of ascites on

November 23, 1992. Approximately 40 mL of ascites fluid was collected between December 3 and 16. Ascites was aliquoted and stored frozen; approximate 50% titer was 200,000-400,000. Antibody was partially purified from 3 mL ascites by ammonium sulfate precipitation on January 21 and further purified by affinity chromatography on protein A on January 26.

4E11. This hybridoma was cloned by limiting dilution on October 9, 1992. Clone plates were examined on October 19, 1992. Cell growth was found in 18/60 wells on the B plate and in 5/60 wells on the C plate. Sixteen of sixteen FGCs were positive for binding on October 20. A total of 11 FGC were tested in competitive inhibition immunoassays between October 27 and November 10. Ten were inhibited by TSA (50 ng/well) and by substrate (1000 ng/well), with little or no product inhibition (50 ng/well) observed under the conditions used. One FGC was negative for binding. Five FGC were isotyped (October 30, November 12) as γ_1, κ. Clone 4E11.C8C was injected into Pristane-primed mice for production of ascites on November 23, 1992. Approximately 18.5 mL of ascites fluid was collected between December 3 and 16. Ascites was aliquoted and stored frozen. Approximate 50% titer was 200,000. Antibody was partially purified from 3 mL of ascites by ammonium sulfate precipitation on January 21 and further purified by affinity chromatography on protein A on January 26.

5B9. This hybridoma was cloned by limiting dilution on October 22, 1992. Clone plates were examined on November 3. Cell growth was found in 30/60 wells on the B plate and in 20/60 wells on the C plate. Four of 50 FGCs were positive for binding on November 4. Three FGC were tested in a competitive inhibition immunoassay on November 10 with the following results: two were inhibited by TSA only; one showed inhibition by substrate (1000 ng/well) in addition to TSA (50 ng/well). Since only 4/50 FGC derived previously were positive for binding this cell line was recloned by limiting dilution on November 6, 1992. Clone plates were examined on November 17; cell growth was observed in 48/60 wells on the B plate and [illegible] 23/30 wells on the C plate. [illegible] tive. Ten FGC were isotyped on November 25 with the result that 9 were positive for [illegible] and 1 was positive for μ, γ_1, κ and λ. The 75 FGC were tested in [illegible]

immunoassays on November 24 and December 1. Forty-nine showed inhibition by TSA only, and 26 showed inhibition by TSA and slight inhibition by substrate. No inhibition by product was observed. Concentrations of inhibitors were 50 ng/well (TSA and product) and 1000 ng/well (substrate). FGC 5B9.C8B was injected into Pristane-primed mice for production of ascites on December 18. Approximately 5.5 mL of ascites fluid was collected between January 3 and January 6. Ascites was aliquoted and stored frozen. Approximate 50% titer of ascites fluid was 100,000. Antibody 5B9.C8B was partially purified from ascites fluid (3 mL) by ammonium sulfate precipitation on January 23 and further purified by affinity chromatography on protein A on January 28.

5E3. This hybridoma was cloned by limiting dilution on October 9, 1992. Clone plates were examined on October 19. Cell growth was found in 14/60 wells on the B plate and in 5/60 wells on the C plate. Twelve of 13 FGC tested on October 20 were positive for binding. A total of 10 FGC were tested in competitive inhibition immunoassays between October 27 and November 12 with the result that inhibition was observed with TSA (50 ng/well) and by substrate (1000 ng/well); little or no inhibition was observed with product (50 ng/well) under the conditions used. Antibodies from three FGC were isotyped (October 30, November 12) as γ_1, κ. Since growth of these clones was relatively sluggish, the parent hybridoma was recloned by limiting dilution on November 6. [The parent line was positive for binding on November 3.] Clone plates were examined on November 17, and cell growth was found in 41/60 wells on the B plate and in 32/60 wells on the C plate. All 73 FGC tested positive for binding on November 18. A total of 72 FGC were tested in competitive inhibition immunoassays (November 24 and December 1), and all were inhibited by TSA (50 ng/well) and, to a lesser extent, by substrate (1000 ng/well). No inhibition was observed with product at 50 ng/well. Ten FGC were isotyped on November 25 as positive for γ_1 and κ. FGC 5E3.D3B was injected [illegible] of ascites on November 23. Approximately 5 mL of ascites was collected between December 3 and December 9, aliquoted and stored frozen. [illegible] was 200,000. Antibody 5E3.D3B was partially purified from

3 mL of ascites by ammonium sulfate precipitation on January 22 and further purified by affinity chromatography on protein A on January 27.

5F4. This hybridoma was cloned by limiting dilution on October 21, 1992. Clone plates were examined on November 2. Cell growth was found in 38/60 wells on the B plate and in 23/60 wells on the C plate. None of the 61 FGCs tested on November 3 were positive for binding. Since the parent line was still positive for binding, it was recloned by limiting dilution on November 5. Since none of the 61 FGC obtained from the initial cloning of this cell line were positive for binding, the parent line was recloned by limiting dilution on November 5, 1992. Clone plates were examined on November 16, and cell growth was found in 1/60 wells on the B plate and in 2/60 wells on the C plate. All 3 of these FGC tested negative for binding on November 17. Since the parent line was still positive for binding (November 17) it was cloned for the third time on November 19. This set of clone plates was examined on November 30, and cell growth was observed in 36/60 wells on the B plate and in 24/60 wells on the C plate. This time, 55/60 FGC tested positive for binding on December 1. These 55 FGC were tested in competitive inhibition immunoassays on December 8 and 15 and showed inhibition by TSA (50 ng/well) and slight inhibition by substrate (1000 ng/well). No inhibition was observed with product at 50 ng/well. Ten FGC were isotyped on December 9 with the results that 7 were positive for γ_1 and κ, 2 were positive for γ_1, γ_{2a} and κ and 1 was positive for γ_1, γ_{2b} and κ. Clone 5F4.B7B was injected into Pristane-primed mice for production of ascites on December 18. Approximately 5.5 mL of ascites fluid was collected between December 30 and January 14. Ascites was aliquoted and stored frozen; approximate 50% titer was 50,000-100,000. Antibody 5F4.B7B was partially purified by ammonium sulfate from 3 mL of ascites on January 23 and further purified by affinity chromatography on protein A on January 28.

[illegible] plates were examined on October 19, and cell growth was found in 2/60 wells on the B plate and 0/60 wells on the C plate. One FGC tested positive [illegible] October 20. The parent line was

recloned on October 21, and the second set of clone plates were examined on November 2. Cell growth was observed in 7/60 wells on the B plate and in 1/60 wells on the C plate. All eight of these FGC were positive for binding on November 3. Six were tested in competitive inhibition immunoassays (November 10 & 12) the results of which indicated inhibition by TSA only under the conditions used. Two FGC were isotyped on November 10: one was positive for γ_1 and κ, and the other was positive for γ_1, γ_{2b}, and κ. Clone 5F11.B3B was injected into Pristane-primed mice for production of ascites on November 23, 1992. Approximately 23.5 mL of ascites was collected between December 23 and January 11. Ascites was aliquoted and stored frozen. Approximate 50% titer was 100,000. Antibody 5F11.B3B was partially purified by ammonium sulfate precipitation from 3 mL ascites on January 23 and further purified by affinity chromatography on protein A (January 28).

5G4. This hybridoma was cloned by limiting dilution on October 22, 1992. Clone plates were examined on November 3. Cell growth was found in 45/60 wells on the B plate and in 22/60 wells on the C plate. Sixty-four of 65 FGCs were positive for binding on November 4. A total of 60 FGC were tested in competitive inhibition immunoassays (November 10 & 12), and 56 were positive for binding and inhibition. Most of these samples were tested under conditions of antibody excess; nevertheless, inhibition was observed with TSA (50 ng/well), substrate (1000 ng/well), and product (50 ng/well). Isotyping was done on supernatants from 11 FGC (November 12). Seven were positive for γ_1 and κ, while four were positive for μ, γ_1, κ and λ. Clone 5G4.F11C was injected into Pristane-primed mice for production of ascites on November 23, 1992. Approximately 21 mL of ascites fluid was collected between December 7 and 16. Ascites was aliquoted and stored frozen. Approximate 50% titer was 200,000. Antibody 5G4.F11C was partially purified by ammonium sulfate precipitation from 3 mL of ascites on January 22 and further purified by affinity chromatography on protein A on January 27.

8F11. This hybridoma was cloned by limiting dilution on October 21, 1992. Clone plates were examined on November 2, and cell growth was observed in 6/60 wells on both the B and C plates. All 12 FGCs were positive for binding on November 3. Seven FGC were tested in a

competitive inhibition immunoassay on November 10. All showed inhibition by TSA at 50 ng/well and by substrate at 1000 ng/well; no product inhibition was observed under the conditions used. Antibodies from 10 FGC were isotyped as γ_1, κ on November 10. Clone 8F11.F3C was injected into Pristane-primed mice for production of ascites on November 23, 1992. Approximately 7.5 mL of ascites was collected between December 3 and 20. Ascites was aliquoted and stored frozen. Approximate 50% titer was 200,000-400,000. Antibody was partially purified by ammonium sulfate precipitation from 3 mL ascites on January 21 and further purified by affinity chromatography on protein A on January 26.

9B10. This hybridoma was cloned by limiting dilution on October 21, 1992. Clone plates were examined on November 2. Cell growth was observed in 42/60 wells on the B plate and in 29/60 wells on the C plate. All 71 FGCs were positive for binding on November 3. Sixty-six FGC were tested in competitive inhibition immunoassays (November 10 & 12); 65 showed inhibition by TSA (50 ng/well) and substrate (1000 ng/well), with slight product inhibition (50 ng/well) observable at low antibody concentrations. Antibodies from 10 FGC were isotyped as positive for γ_1 and κ on November 10. Clone 9B10.B7C was injected into Pristane-primed mice for production of ascites on November 23, 1992. Approximately 8.5 mL of ascites fluid was collected between December 4 and 9. Ascites was aliquoted and stored frozen. Approximate 50% titer was 400,000. Antibody was partially purified by ammonium sulfate precipitation from 3 mL ascites on January 22 and further purified by affinity chromatography on protein A on January 27.

9C5. This hybridoma was cloned by limiting dilution on October 21, 1992. Clone plates were examined on November 2, and cell growth was observed in 15/60 wells on the B plate and in 12/60 wells on the C plate. Seven of 27 FGCs tested positive for binding on November 3. These FGC were analyzed in a competitive inhibition immunoassay on November 10 with the result that all were inhibited by TSA (50 ng/well) and by substrate (1000 ng/well); a small amount of product inhibition was observed at a concentration of 50 ng/well. Antibodies from these 7 clones were isotyped as positive for γ_1 and κ on

November 10. Since the per cent of FGC which were positive for binding was low and the parent line was still positive for binding (November 3), it was recloned by limiting dilution on November 5. Clone plates were examined on November 16, and cell growth was observed in 51/60 wells on the B plate and in 44/60 wells on the C plate. All 95 FGC were positive for binding when tested on November 17. A total of 91 FGC were tested in competitive inhibition immunoassays (November 24 and December 1). Eighty-three showed inhibition by TSA at 50 ng/well and slight inhibition by substrate at 1000 ng/well; the other 8 showed inhibition by TSA but not by substrate. None were inhibited by product at 50 ng/well. Ten FGC were isotyped on November 25 as positive for γ_1 and κ. Clone 9C5.E7C was injected into Pristane-primed mice for production of ascites on January 27. Approximately 24 mL of ascites fluid was collected between February 3 and 12. Ascites was aliquoted and stored frozen; average 50% titer was 200,000. Antibody was purified from 3 mL of ascites by ammonium sulfate precipitation on February 24 followed by affinity chromatography on protein A (February 25).

9C10. This hybridoma was cloned by limiting dilution on October 22, 1992, and clone plates were examined on November 3. Cell growth was observed in 13/60 wells on the B plate and in 5/60 wells on the C plate. Twelve of 18 FGCs were positive for binding on November 4. A total of eight FGC were tested in competitive inhibition immunoassays on November 10 and 12 with the result that, for seven clones, inhibition was observed with TSA (50 ng/well) and substrate (1000 ng/well); slight product inhibition was observed (50 ng/well). One FGC appeared to be inhibited by TSA only. Antibodies from six FGC were isotyped as γ_1, κ on November 12. Clone 9C10.F8B was injected into Pristane-primed mice for production of ascites on November 23, 1992. Approximately 24 mL of ascites fluid was collected between December 4 and 23. Ascites was aliquoted and stored frozen. Approximate 50% titer was 200,000-400,000. Antibody was partially purified by ammonium sulfate precipitation from 3 mL of ascites on January 21 and further purified by affinity chromatography on protein A on January 26.

10B6. This hybridoma was cloned by limiting dilution on October 22, 1992. Clone plates were examined on November 3. Cell growth was observed in 15/60 wells on the B plate and in 6/60 wells on the C plate. None of the 21 FGCs was positive for binding when tested on November 4, 1992. The parent line was negative for binding on November 3, and this hybridoma was not recloned.

10C10. This hybridoma was cloned by limiting dilution on October 22, 1992. Clone plates were examined on November 3, 1992. Cell growth was observed in 22/60 wells on the B plate and in 13/60 wells on the C plate. All 31 FGCs tested for binding on November 4 were positive. A total of 13 of these FGC were tested in competitive inhibition immunoassays on November 10 and 12 and showed inhibition by TSA (50 ng/well) and substrate (1000 ng/well). Slight product inhibition was also observed at a concentration of 50 ng/well. Antibodies from ten FGC were isotyped as positive for γ_1 and κ on November 10 and 12. Clone 10C10.C10C was injected into Pristane-primed mice for production of ascites on November 23, 1992. Approximately 4 mL of ascites fluid was collected between December 9 and 16. Approximate 50% titer was 200,000; aliquots were stored frozen. Approximately 5 mL of ascites fluid was collected from a second mouse during this same period. This material tested negative for activity. Because of this 10C10.C10C was injected into an additional primed mouse on January 27, 1993. Approximately 3 mL of ascites fluid was collected between February 3 and 8. Ascites was aliquoted and stored frozen. This batch of ascites also had little binding activity, with approximate 50% titers of 100. Antibody was partially purified by ammonium sulfate precipitation from 3 mL of ascites (first mouse) on January 22 and further purified by affinity chromatography on protein A on January 27.

11B6. This hybridoma was cloned by limiting dilution on October 22, 1992. Clone plates were examined on November 3, and growth was observed in 23/60 wells on the B plate and 17/60 wells on the C plate. Twenty-nine of 30 FGCs tested on November 4 were positive for binding. A total of 16 FGC were analyzed in competitive inhibition immunoassays (November 10 & 12). Thirteen showed inhibition by TSA (50 ng/well) and substrate

(1000 ng/well) but not by product (50 ng/well). Three clones were tested in antibody excess and showed only partial inhibition by TSA and no inhibition by substrate and product. Antibodies from ten FGC were isotyped on November 10; 9 were positive for γ_{2b} and κ, while one was positive for γ_{2b}, γ_1, and κ. Clone 11B6.D8C was injected into Pristane-primed mice for production of ascites on November 23, 1992. Approximately 6.5 mL of ascites was collected between December 9 and 16. Ascites was aliquoted and stored frozen. Approximate 50% titer was 200,000. Antibody was partially purified by ammonium sulfate precipitation from 3 mL of ascites on January 21 and further purified by affinity chromatography on protein A on January 26.

11C6. This hybridoma was cloned by limiting dilution on October 9, 1992. Clone plates were examined on October 19; cell growth was observed in 5/60 wells on the B plate and in 3/60 wells on the C plate. Four of six FGCs were positive for binding on October 20. One clone was tested in a competitive inhibition immunoassay on November 3; binding was inhibited by TSA at 50 ng/well and substrate at 1000 ng/well. No product inhibition was observed at a concentration of 50 ng/well. Cells from the 11C6 line grew poorly, so it was decided to reclone the parent hybridoma on November 6. [The parent line tested positive for binding on November 3.] Clone plates were examined on November 17, and no cell growth was observed. Therefore the cell line was cloned by limiting dilution on November 19. Clone plates were examined on November 30. Cell growth was observed in 17/60 wells on the B plate and in 7/60 wells on the C plate. FGC were tested for binding on December 1, and 23/24 were positive. A total of 22 FGC were tested in competitive inhibition immunoassays (December 8 and 15) with the result that binding was inhibited by TSA at 50 ng/well and slightly inhibited by substrate at 1000 ng/well. No product inhibition was observed at 50 ng/well. Eight FGC were isotyped on December 9 as positive for γ_{2b} and κ. Clone 11C6.F8C was injected into Pristane-primed mice for production of ascites on December 18. Approximately 21.5 mL of ascites fluid was collected between December 30 and January 11. Ascites was aliquoted and frozen. Average 50% titer was 50,000-100,000. Antibody

11C6.F8C was partially purified from 3 mL of ascites by ammonium sulfate precipitation on January 22 and further purified by affinity chromatography on protein A on January 27.

11D5. This hybridoma was cloned by limiting dilution on October 9, 1992. Clone plates were examined on October 19. Cell growth was observed in 11/60 wells on the B plate and in 4/60 wells on the C plate. None of the 15 FGCs tested on October 20 were positive for binding. The parent hybridoma was recloned on October 21, and the second set of clone plates was examined on November 2. Growth was observed in 4/60 wells on the B plate and in 2/60 wells on the C plate. None of these six FGC tested positive for binding on November 3. The parent line was also negative for binding on November 3, so no further work was done with this cell line.

11D9. This hybridoma was cloned by limiting dilution on October 9, 1992. Clone plates were examined on October 19, and cell growth was observed in 3/60 wells on the B plate and in 1/60 wells on the C plate. None of the four FGCs tested on October 20 was positive for binding. The parent line was positive for binding on October 21 and was recloned on that date. The second set of cloning plates was examined on November 2. Cell growth was observed in 24/60 wells on the B plate and in 13/60 wells on the C plate. None of these 37 FGC was positive for binding when tested on November 3. Since the parent line was also negative on November 3, no further work was done with this cell line.

11F3. This hybridoma was cloned by limiting dilution on October 9, and clone plates were examined on October 19. Cell growth was observed in 15/60 wells on the B plate and in 16/60 wells on the C plate. On October 20, 23/25 FGCs were positive for binding. Twelve FGC were tested in competitive inhibition immunoassays between October 27 and November 12. Eleven showed inhibition by TSA (50 ng/well) and substrate (1000 ng/well), with no product inhibition under the conditions used. One FGC was negative. Antibodies from seven clones were isotyped as γ_1, κ (October 30 and November 12). Clone 11F3.E4C was injected into Pristane-primed mice for production of ascites on November 23, 1992. Approximately 12.5 mL of ascites was collected between December 3 and 10; ascites was aliquoted

and stored frozen. Average 50% titer was 100,000. Antibody was purified by ammonium sulfate precipitation from 3 mL of ascites on January 22 and further purified by affinity chromatography on protein A on January 27.

12B11. This hybridoma was cloned by limiting dilution on October 9. Clone plates were examined on October 19. Cell growth was observed in 2/60 wells on the B plate and 6/60 wells on the C plate. None of the eight clones were positive for binding (October 20). The parent line was recloned on October 21, and the second set of clone plates examined on November 2. Cell growth was observed in 29/60 wells on the B plate and in 14/60 wells on the C plate. One of these FGCs (12B11.F2B) was positive for binding on November 3, but showed no inhibition by TSA when tested in a competitive inhibition immunoassay on November 10. Since the parent line was still positive for binding on November 3, it was cloned again on November 5. Clone plates were examined on November 16 with the result that cell growth was observed in 2/60 wells on the B plate and in 5/60 wells on the C plate. Six FGC from this cloning were tested in a competitive inhibition immunoassay on December 1; all were inhibited by TSA (50 ng/well) and substrate (1000 ng/well). The 6 FGC were isotyped on December 4 as positive for γ_{2a} and κ. Since we had gotten variable results with cloning of this hybridoma, we cloned for the fourth time on November 19. Clone plates were examined on November 30 with the result that cell growth was observed in 15/60 wells on the B plate and in 9/60 wells on the C plate. Twenty-one of 24 FGC from this cloning tested positive for binding on December 1. These 21 FGC were tested in competitive inhibition immunoassays (December 8 and 15) with the result that 20 were inhibited by TSA (50 ng/well) and slightly inhibited by substrate (1000 ng/well). One was inhibited by TSA only. No product inhibition was observed (50 ng/well). Ten FGC were isotyped on December 9 with the result that 6 were positive for γ_{2a} and κ and 4 were positive for γ_{2a}, γ_1 and κ. Clone 12B11.C5C was injected into Pristane-primed mice for production of ascites on December 18. Approximately 12 mL of ascites was collected between December 30 and January 5. Ascites was aliquoted and stored frozen. Average 50% titer was 50,000-100,000. Antibody was purified by ammonium sulfate

precipitation from 3 mL of ascites on January 23 and further purified by affinity chromatography on protein A on January 28.

13E11. This hybridoma was cloned by limiting dilution on October 9, 1992. Clone plates were examined on October 19, and cell growth was observed in 4/60 wells on the B plate and 6/60 wells on the C plate. All four FGCs tested on October 20 were positive for binding. A total of eight FGC were analyzed in competitive inhibition immunoassays between October 27 and November 12 with the result that inhibition was observed with TSA (50 ng/well) and substrate (1000 ng/well), with little/no product inhibition (at 50 ng/well). Antibodies from two of these clones were isotyped as γ_1, κ (October 30, November 12). Since antibodies from this hybridoma line looked promising even though cell growth was generally poor, the parent hybridoma was recloned by limiting dilution on November 5. Clone plates were examined on November 16 with the result that cell growth was observed in 15/60 wells on the B plate and in 14/60 wells on the C plate. All 29 FGC tested positive for binding on November 17. FGC were analyzed in competitive inhibition immunoassays (November 24 and December 1). All were inhibited by TSA at 50 ng/well and 23/24 showed slight substrate inhibition at 1000 ng/well. No product inhibition was observed (50 ng/well). Ten FGC were isotyped on November 25 as positive for γ_1 and κ. Clone 13E11.B6B was injected into Pristane-primed mice for production of ascites on November 23. Approximately 24.5 mL of ascites fluid was collected between December 4 and 24. Ascites was aliquoted and stored frozen. Average 50% titer was 200,000-400,000. Antibody was partially purified by ammonium sulfate precipitation from 3 mL of ascites on January 21 and further purified by affinity chromatography on protein A on January 26.

14F4. This hybridoma was cloned by limiting dilution on October 9, 1992. Clone plates were examined on October 19. Cell growth was observed in 10/60 wells on the B plate and in 2/60 wells on the C plate. Only 1/11 clones was positive for binding (October 20, November 3), and this clone died. The parent hybridoma was racloned on October 21, and the second set of clone plates examined on November 2. Cell growth was observed in 9/60 wells

on the B plate and in 2/60 wells on the C plate. None of these clones were positive for binding (November 3). Since the parent line still tested positive on November 3, it was cloned again on November 5. Clone plates were examined on November 16. Cell growth was observed in 8/60 wells on the B plate and in 7/60 wells on the C plate. All 15 FGC were negative when tested on November 17. Since the parent line tested positive for binding on November 17, it was cloned again on November 19. Clone plates were examined on November 30 with the result that cell growth was observed in 53/60 wells on the B plate and in 35/60 wells on the C plate. One of 88 FGC tested positive for binding on December 1. This clone (14F4.C11B) was inhibited by TSA and by substrate in a competitive inhibition immunoassay (December 8) and was isotyped as positive for μ, γ_1, κ and λ. 14F4.C11B was subcloned by limiting dilution on December 17. Subcloning plates were examined on December 28, and cell growth was observed in 40/60 wells on the B plate and in 35/60 wells on the C plate. SGC were screened on December 29 and January 6 with the result that 29/75 were positive for binding. A total of 30 SGC were tested in competitive inhibition immunoassays (January 7 and 12) with the result that 25 were inhibited by TSA (50 ng/well) and by substrate (1000 ng/well) and 5 were negative. Twenty-four SGC were isotyped with the results that 3 were positive for γ_1 and κ, 1 was positive for μ, γ_1, κ and λ, 10 were positive for μ, γ_{2a}, κ and λ, 8 were positive for γ_{2a} and κ and 2 were positive for μ, γ_1, γ_{2a}, κ and λ. Clone 14F4.C11B.E2B injected into Pristane-primed mice for production of ascites on January 27. Approximately 19 mL of ascites fluid was collected between February 8 and 15. Ascites was aliquoted and stored frozen; average 50% titer was 100,000-200,000. Antibody was purified by ammonium sulfate precipitation from 3 mL of ascites on February 24 and by affinity chromatography on protein A (February 25).

6.3 Hybridomas from F103092

12F5. This hybridoma culture was cloned by limiting dilution on December 17, 1992. Clone plates were examined on December 28. Cell growth was found in 24/60 wells on the B plate and in 15/60 wells on the C plate. In an initial screening assay, 31/31 FGC were positive for binding. Thirty-eight FGC were tested in competitive inhibition immunoassays (January 7

and 12), and all were inhibited by TSA at 50 ng/well and by substrate at 1000 ng/well. No inhibition by product (50 ng/well) was observed. Fifteen FGC were isotyped on January 12 as positive for γ_{2a} and κ. Clone 12F5.C9B was injected into Pristane-primed mice for production of ascites on January 27. Approximately 12.5 mL of ascites fluid was collected between February 4 and 11. Ascites was aliquoted and stored frozen; average 50% titer was 200 000. Antibody was purified by ammonium sulfate precipitation from 3 mL of ascites on February 24 followed by affinity chromatography on protein A on February 25.

15E6. This hybridoma was cloned by limiting dilution on December 17, 1992. Clone plates were examined on December 28, and cell growth was observed in 28/60 wells on the B plate and in 15/60 wells on the C plate. In an initial screening assay (December 29), 27/31 FGC were positive for binding. A total of 39 FGC were tested in competitive inhibition immunoassays (January 7 and 12) with the result that 36 were inhibited by TSA at 50 ng/well and by substrate at 1000 ng/well (3 were negative for binding). Fifteen FGC were isotyped on January 12 as positive for γ_{2a} and κ. Clone 15E6.E11B was injected into Pristane-primed mice for production of ascites on January 27. Approximately 24.5 mL of ascites was collected between February 3 and 10. Ascites was aliquoted and stored frozen; average 50% titer was 100,000-200,000. Antibody was purified by ammonium sulfate precipitation from 3 mL of ascites on February 24 followed by affinity chromatography on protein A (February 25).

This hybridoma was also cloned on December 17 in the absence of peritoneal exudate feeder cells (15E6.2). Clone plates were examined on December 28, and cell growth was observed in 44/60 wells on the B plate and in 40/60 wells on the C plate. On initial screening (December 29) 73/73 FGC were positive for binding. All 84 FGC were tested in competitive inhibition immunoassays (January 7 and 12) with the result that all were inhibited by TSA at 50 ng/well and by substrate at 1000 ng/well. Fifteen FGC were isotyped on January 12 as positive for γ_{2a} and κ. Clone 15E6.2.D4C was injected into Pristane-primed mice on January 27 for production of ascites. Approximately 7 mL of ascites fluid was collected

between February 8 and 10. Ascites was aliquoted and stored frozen; average 50% titer was 200,000. [Antibody was not purified.]

7.0 Tables

TABLE I
SCHEDULE FOR IMMUNIZATION OF MICE (GROUPS 1 AND 2) WITH METHOXYSTILBENE-PROTEIN CONJUGATES

Day	Date	Procedure
-1	03-31-92	Preimmune bleed and ear tag
0	04-01-92	Primary immunization[a] of mice
14	04-15-92	Bleed 1 of mice
28	04-29-92	Boost mice[b]
35	05-06-92	Bleed 2 of mice
40	05-11-92	I.V. boost selected mouse
42	05-13-92	Boost mice[b]
43	05-14-92	Fusion
49	05-20-92	Bleed 3 of mice
56	05-27-92	Boost mice[b]
63	06-03-92	Bleed 4 of mice
70	06-10-92	Boost mice[b]
77	06-17-92	Bleed 5 of mice
84	06-24-92	Boost mice[b]
91	07-06-92	Bleed 6 of mice

[a] 50 µg immunogen and 50 µL Bordetella pertussis in CFA, injected ip.
[b] 25 µg immunogen in IFA, injected ip.

TABLE II
SCHEDULE FOR IMMUNIZATION OF MICE (GROUP 3) WITH METHOXYSTILBENE-BSA CONJUGATE

Day	Date	Procedure
-2	08-03-92	Preimmune bleed; ear-tag
0	08-05-92	Primary immunization
14	08-19-92	Bleed 1
28	09-02-92	Boost
35	09-09-92	Bleed 2
42	09-16-92	Boost
49	09-23-92	Bleed 3
56	09-30-92	Boost
63	10-07-92	Bleed 4

TABLE III

A. APPROXIMATE 50% TITERS OF SERA FROM MICE (GROUPS 1 AND 2) IMMUNIZED WITH METHOXYSTILBENE-PROTEIN CONJUGATES

Group 1		
Mouse #	Bleed 1	Bleed 2
101	2,500	5,000
102	2,500	5,000
103	5,000	>20,000
104	5,000	>20,000
105	<100	5,000
106	20,000	>20,000
107	2,500	20,000
108	5,000	>20,000
109	2,500	20,000
110	2,500	10,000
111	5,000	>20,000
112	5,000	>20,000

Group 2		
Mouse #	Bleed 1	Bleed 2
113	<100	20,000
114	<100	1,000
115	<100	20,000
116	<100	20,000
117	<100	20,000
118	<100	20,000
119	<100	>20,000
120	1,000	20,000
121	<100	<100
122	<100	20,000
123	<100	>20,000
124	<100	10,000

B. APPROXIMATE 50% TITERS OF SERA FROM MICE (GROUP 3) IMMUNIZED WITH METHOXYSTILBENE-BSA CONJUGATE

Mouse #	Bleed 1	Bleed 2	Bleed 3	Bleed 4
125	<100	>20,000	–	–
126	<100	20,000	>20,000	>20,000
127	<100	>20,000	>20,000	>20,000
128	<100	>20,000	>20,000	>20,000
129	1000	>20,000	>20,000	(died)
130	100	20,000	>20,000	>20,000
131	1000	>20,000	>20,000	20,000
132	1000	>20,000	>20,000	>20,000
133	<100	1000	>20,000	>20,000
134	1000	>20,000	>20,000	>20,000
135	<100	>20,000	>20,000	>20,000
136	<100	>20,000	>20,000	>20,000

TABLE IV
AFFINITY CHROMATOGRAPHY ON PROTEIN A

Antibody	Isotype	Protein Applied (mg)	Protein Recovered (mg)	Protein in Eluate (mg)	% Activity in Eluate
1F4.C11C	γ_1,κ	26.1	23.5	8.5	38
1G9.F3B	γ_1,κ	28.0	28.8	10.1	38
4C5.D10C	γ_1,κ	11.8	12.4	6.6	57
4E11.C8C	γ_1,κ	7.6	7.0	3.2	100
5B9.C8B	γ_1,κ	17.4	18.5	8.5	27
5C10.E4B	γ_1,κ	4.4	4.8	1.3	65
5E3.D3B	γ_1,κ	16.8	15.2	8.2	63
5F4.B7B	γ_1,κ	16.8	26.4	9.2	25
5F11.B3B	γ_1,κ	26.7	20.4	9.4	32
5G4.F11C	γ_1,κ	28.8	30.5	8.8	8
8F11.F3C	γ_1,κ	8.0	7.0	2.4	100
9B10.B7C	γ_1,κ	9.0	7.7	3.3	100
9C5.E7C	γ_1,κ	11.4	11.2	4.3	100
9C10.F8B	γ_1,κ	7.3	6.9	3.6	100
10C10.C10C	γ_1,κ	17 5	10.7	6.2	92
10D6.B3C.D4C	γ_{2a},κ	13.6	14.9	5.5	100
11B6.D8C	γ_{2b},κ	9.3	9.7	2.4	100
11C6.F8C	γ_{2b},κ	19.5	18.2	6.7	48
11F3.E4C	γ_1,κ	13.0	17.5	7.7	25
12B11.C5C	γ_{2a},κ	7.0	6.2	2.9	100
12F5.C9B	γ_{2a},κ	27.3	25.7	12.6	68
13E11.B6B	γ_1,κ	12.6	9.2	4.5	100
14E9.D2B	γ_{2b},κ	11.8	20.7	8.9	83
4F4.C11B.E2B	γ_{2a},κ	24.6	25.9	14.2	83
15E6.E11B	γ_{2a},κ	18.6	19.7	11.8	100
19E2.G5B	γ_3,λ	6.0	9.7	2.8	100
20C10.D11B	γ_1,κ	7.0	5.6	1.0	66
20F2.C2B	γ_1,κ	1.6	2.5	0.4	66

TABLE V
CHARACTERIZATION OF ANTIBODIES

Antibody	Isotype	IC50 (ng/well) TSA	IC50 (ng/well) Substrate	IC50 (ng/well) Product	Velocity Ratio**
15E6.E11B	γ_{2a},κ	0.0029	5.30	ND*	1.0
14F4.C11B.E2B	γ_{2a},κ	0.0040	2.39	ND	0.9
12F5.C9B	γ_{2a},κ	0.0075	70	ND	0.9
9C10.F8B	γ_1,κ	0.018	1000-3000	34	1.5
5F4.B7B	γ_1,κ	**0.018**	**3000**	**>>3000**	**4.9**
5G4.F11C	γ_1,κ	**0.021**	**3000**	**>>3000**	**5.9**
11F3.E4C	γ_1,κ	**0.022**	**3000**	**>>3000**	**4.6**
9C5.E7C	γ_1,κ	0.023	1000-3000	18.7	1.1
13E11.B6B	γ_1,κ	0.027	1000-3000	26	1.4
1G9.F3B	γ_1,κ	0.031	>3000	>3000	1.3
5F11.B3B	γ_1,κ	**0.035**	**3000**	**>>3000**	**4.2**
5B9.C8B	γ_1,κ	0.040	>>3000	>3000	1.7
4C5.D10C	γ_1,κ	0.078	>3000	>3000	1.4
8F11.F3C	γ_1,κ	0.114	>3000	ND	1.2
9B10.B7C	γ_1,κ	0.128	3000	ND	1.6
1F4.C11C	γ_1,κ	1.34	918	ND	1.8
5E3.D3B	γ_1,κ	1.38	700	ND	0.8
4E11.C8C	γ_1,κ	4.08	618	ND	1.3
12B11.C5C	γ_{2a},κ	7.30	584	ND	2.0
11C6.F8C	γ_{2b},κ	19.2	>>3000	ND	1.1

* ND - Not Done

** Ratio of initial velocity with antibody to initial background velocity (200 mM enone, 10 mM KCN, 20 mM antibody at 37°C and pH 7.4).

TABLE VI

Hybridoma/Antibody	Volume	Dates Collected	Average 50% Titer
	Ascites Collected--F051492		
5C10.E4B	10 mL	07/29-08/07/92	50,000-100,000
8F5.E3C	6 mL	07/29-31/92	100
10D6.B3C	6 mL	07/29-31/92	50,000-100,000
10D6.B3C.D4C	11 mL	08/29-09/03/92	50,000-100,000
14E9.D2B	7 mL	07/28-29/92	50,000-100,000
14E9.D2B.E8B	9 mL	08/28-09/06/92	10,000-50,000
19E2.G5B	3.5 mL	09/01-06/92	10,000-50,000
20C10.D11B	6 mL	07/26/92	50,000-100,000
20F2.C10B	8 mL	07/28-31/92	10,000-50,000
	Ascites Collected--F091792		
1F4.C11C	13 mL	12/30/92-1/11/93	50,000-100,000
1G9.F3B	11.5 mL	12/31/92-1/14/93	50,000-100,000
4C5.D10C	40 mL	12/3-12/16/92	200,000-400,000
4E11.C8C	18.5 mL	12/3-12/16/92	200,000
5B9.C8B	5.5 mL	1/3-1/6/93	100,000
5E3.D3B	6 mL	12/3-12/9/92	200,000
5F4.B7B	5.5 mL	12/30/92-1/14/93	50,000-100,000
5F11.B3B	23.5 mL	12/23/92-1/11/93	100,000
5G4.F11C	21 mL	12/7-12/16/92	200,000
8F11.F3C	7.5 mL	12/3-12/20/92	200,000-400,000
9B10.B7C	8.5 mL	12/4-12/9/92	400,000
9C5.E7C	24 mL	2/3-2/12/92	200,000
9C10.F8B	24 mL	12/4-12/23/92	200,000-400,000
10C10.C10C	4 mL	12/9-12/16/92	200,000
	5 mL	12/9-12/16/92	~100
	3 mL	2/3-2/8/93	~100
11B6.D8C	6.5 mL	12/9-12/16/92	200,000
11C6.F8C	21.5 mL	12/30/92-1/11/93	50,000-100,000
11F3.E4C	12.5 mL	12/3-12/10/92	100,000
12B11.C5C	12 mL	12/30/92-1/5/93	50,000-100,000
13E11.B6B	24.5 mL	12/4-12/24/92	200,000-400,000
14F4.C11B.E2B	19 mL	2/8-2/15/93	100,000-200,000
	Ascites Collected--F103092		
12F5.C3B	12.5 mL	2/4-2/11/93	200,000
15E6.E11B	24.5 mL	2/3-2/10/93	100,000-200,000
15E6.2.D4C	7 mL	2/8-2/10/93	200,000

TABLE VII
STANDARD CALIBRATION CURVES

Date	Slope (μV-sec/μM)	y-intercept (μV-sec)	r^2
2/3/93	129531	-9815	0.998
2/4/93	125545	30765	0.997
2/9/93	135742	-97056	0.999
2/11/93	138574	-123579	0.999
2/15/93	118389	-27652	1.000
2/17/93	119137	-20252	1.000
2/18/93	118230	-21244	1.000
2/19/93	119489	-33971	1.000
2/23/93	121515	-25645	1.000
2/24/93	122035	-45976	1.000
2/25/93	121701	-48203	1.000
2/26/93	122463	-54588	1.000
3/5/93	70538	-1778	1.000
3/8/93	66792	26696	1.000
3/9/93	69403	14250	1.000
3/10/93	72160	6479	1.000
3/11/93	72591	-27057	1.000
3/12/93	71951	-1822	1.000
3/15/93	52987	15706	1.000
3/22/93	54022	9661	1.000
3/23/93	54846	9227	1.000
3/24/93	54513	2761	1.000
4/1/93	60633	-6264	1.000
4/2/93	60435	-3790	1.000
4/5/93	62484	-11951	1.000
4/6/93	62732	-6274	1.000
4/7/93	61830	-283.6	1.000
4/[illegible]93	63703	-12741	1.000
4/12/93	64861	-7961	1.000
4/13/93	67301	-17461	1.000
4/14/93	57994	37934	1.000
4/15/93	57718	8152	1.000
4/16/93	59160	0.0	1.000
4/19/93	60418	-1360	1.000
4/28/93	60334	85.0	1.000
4/29/93	58167	13450	1.000
4/30/93	59348	3610	1.000

TABLE VIII
SCREENED CELL LINES

Name	$v_{o,\ Bkg}$ ($\mu M\ h^{-1}$)	$v_{o,\ Ab}$ ($\mu M\ h^{-1}$)	$v_{o,\ Ab}/v_{o,\ Bkg}$
5F11.B3B	2.423	10.213	4.215
5F4.B7B	2.318	10.700	4.616
5B9.C8B	2.326	3.902	1.677
1G9.F3B	2.197	2.869	1.306
12B11.C5C	2.340*	4.679	1.999
11B6.D8C	1.916	2.408	1.257
8F11.F3C	2.527	3.043	1.204
14E9.D2B	2.317*	2.003	0.864
4E11.C8C	2.317*	3.090	1.334
5E3.D3B	2.317*	1.957	0.845
14F4.C11B.E2B	2.317*	2.126	0.918
15E6.E11B	2.317*	2.326	1.004
9C5.E7C	2.317*	2.535	1.094
12F5.C9B	3.831	3.322	0.867
10C10.C10C	3.831	3.730	0.974
11F3.E4C	3.831	17.818	4.623
11C6.F8C	3.831	4.071	1.063
1F4.C11C	2.114	3.812	1.803
4C5.D10C	2.114	2.869	1.357
5G4.F11C	2.422	14.273	5.893
13E11.B6B	1.973	2.726	1.382
9B10.B7C	1.973	3.135	1.589
9C10.F8B	2.012	2.949	1.466
5C10.E4B	0.954	0.706	0.740
10D6.B3C.D4C	0.954	2.38	2.49
19E2.G5B	0.954	1.24	1.30
20C10.D11B	0.954	0.273	0.286
20F2.C2B	0.954	1.33	1.39

* Average $v_{o,\ Bkg}$

TABLE IX
LINEWEAVER-BURK DATA
ANTIBODY 5G4.F11C

[KCN] (mM)	[enone] (μM)	v_o, Bkg (μM h^{-1})	v_o, Ab (μM h^{-1})	v_o (Ab-Bkg) (μM h^{-1})
10	50	0.587	6.170	5.583
10	100	0.956	9.230	8.274
10	150	1.663	11.016	9.353
10	200	1.981	12.462	10.481
5	50	0.284	3.795	3.511
5	100	0.622	5.103	4.481
5	150	0.843	6.506	5.663
5	200	1.102	6.757	5.655
2	100	0.271	2.497	2.226
2	150	0.399	2.913	2.514
2	200	0.573	3.416	2.843

TABLE X

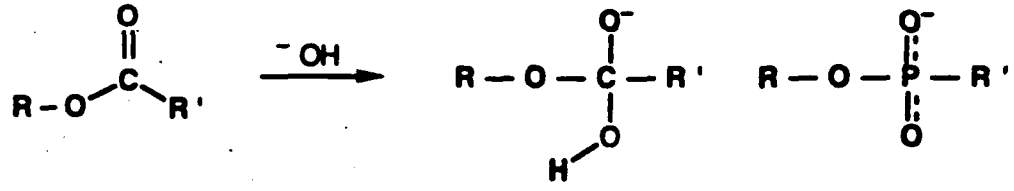
Animal #	Dose	Initial Weight	Final Weight	Comments
1	200 mg/kg	20.6 g	19.8 g	None
2	200 mg/kg	26.0 g	25.5 g	None
3	200 mg/kg	27.0 g	24.9 g	None
4	200 mg/kg	23.1 g	20.6 g	1*
5	135 mg/kg	25.2 g	24.8 g	2*
6	135 mg/kg	22.8 g	23.0 g	2*
7	90 mg/kg	23.7 g	24.1 g	None
8	90 mg/kg	24.5 g	23.6 g	None
9	60 mg/kg	27.2 g	27.1 g	None
10	60 mg/kg	24.0 g	23.7 g	None
11	0 mg/kg	24.8 g	24.2 g	None
12	0 mg/kg	25.4 g	25.2 g	None

*1. This animal died 21-23 hours after administration of test compound. On necropsy, a puncture was found in the esophagus and blood had collected in the tissues around the neck. These findings are consistent with death as a result of a technical error in administration of the test compound (gavage error).

*2. These animals were not very active for approximately 1 hour after administration of test compound.

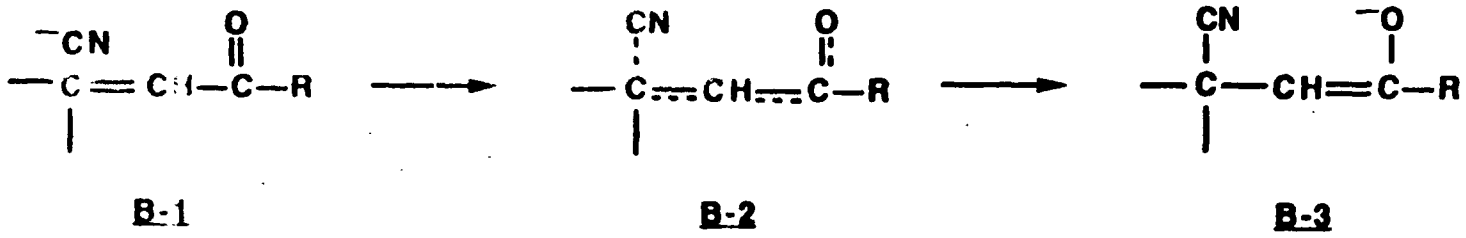
8.0 Charts

CHART A
ESTER HYDROLYSIS AND TRANSITION STATE ANALOG

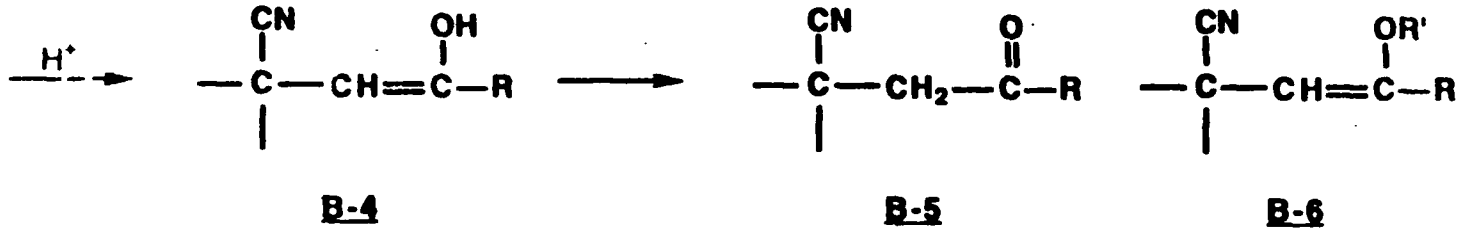


A-1 A-2 A-3

CHART B
REACTION PATHWAY TO HYDROCYANATION OF ENONES

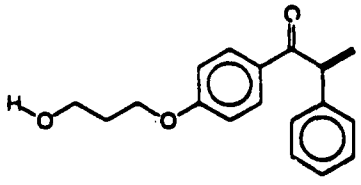


B-1 B-2 B-3

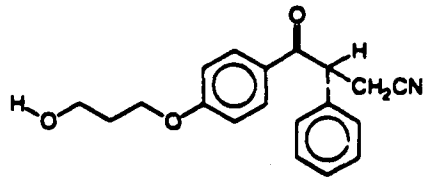


B-4 B-5 B-6

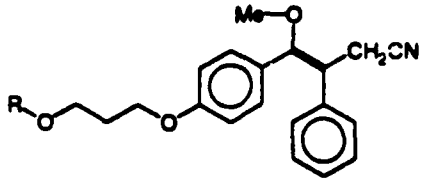
CHART C. Only *E*-isomer of C-1 is shown. However, the material was an approximately 1:1 mixture of the *E*- and *Z*-isomers.



C-2



C-3



C-1 a. R = OAc
b. R = OH
c. R = CO–N
d. R = Protein—NH
e. R = Me_2N

Chart D. Diagram of equilibria involved in the antibody catalyzed reaction of cyanide with an unsaturated ketone (enone) in a random sequential bireactant fashion (adapted from Segel, 1975). The factor a is the factor for the effect of the binding of one substrate on the dissociation constant of the other. The cyanide, represented by CN, may be CN^- or HCN.

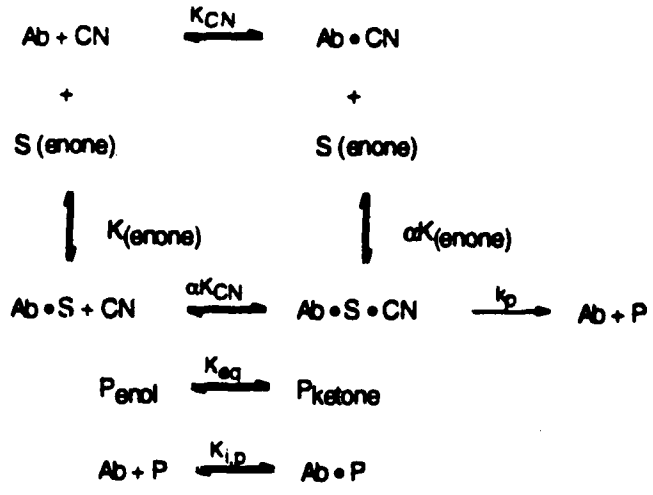
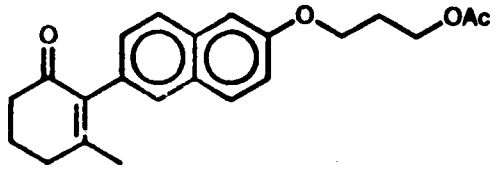


CHART E

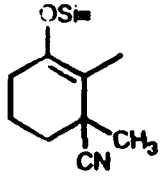


1

+ 2 Et_2AlCN → 3 $(EtO)_2P(O)Cl$ → X no vinyl phosphate

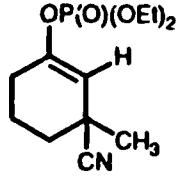
→

→



2

+ $(EtO)_2P(O)Cl$ + $(n\text{-}Bu)_4NF$ $\xrightarrow[THF]{-78° \rightarrow r.t.}$ X



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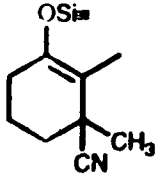
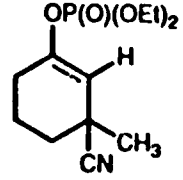


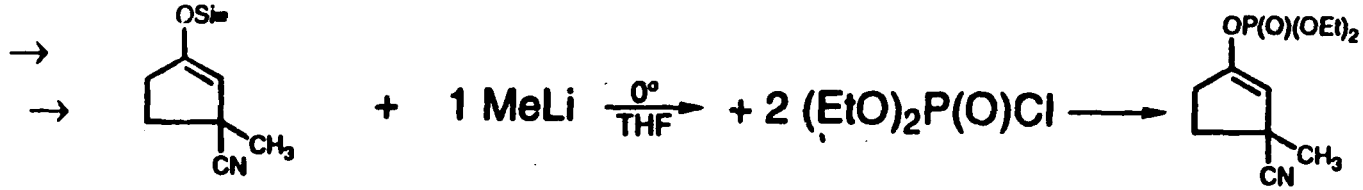
2

+ 1.1 $(nBu)_4NF$ $\xrightarrow[THF]{-78°}$ + 1.5 $(EtO)_2P(O)Cl$ ⟶ X

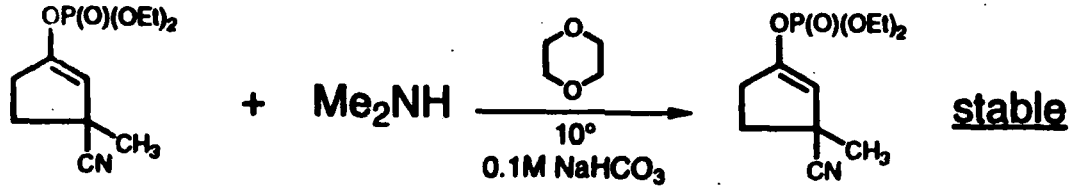


4

CHART F

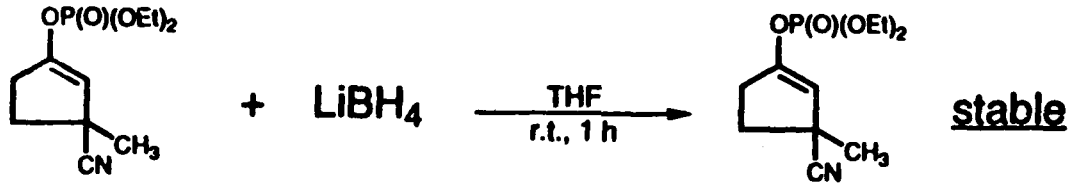


1 2



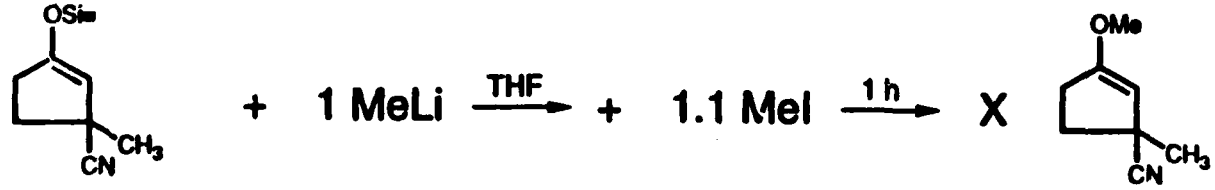
stable

2 2



stable

2 2



3 4

CHART G

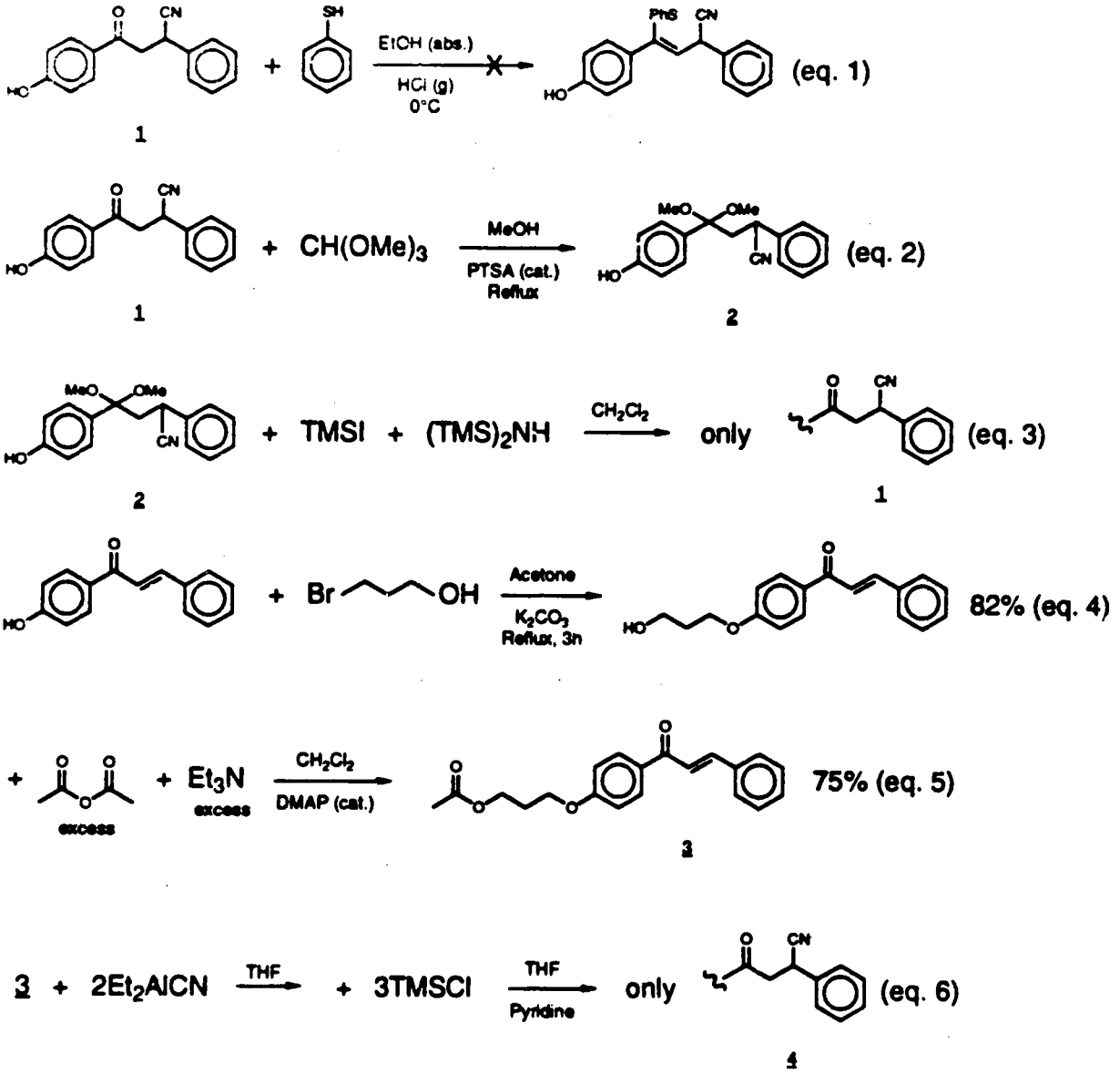

EtOH (abs.)
HCl (g)
0°C
(eq. 1)
1
MeOH
PTSA (cat.)
Reflux
CH(OMe)3
(eq. 2)
2
TMSI
(TMS)2NH
CH2Cl2
only
(eq. 3)
Acetone
K2CO3
Reflux, 3h
82% (eq. 4)
Et3N
excess
DMAP (cat.)
75% (eq. 5)
3
2Et2AlCN
THF
3TMSCl
Pyridine
(eq. 6)
4

CHART H

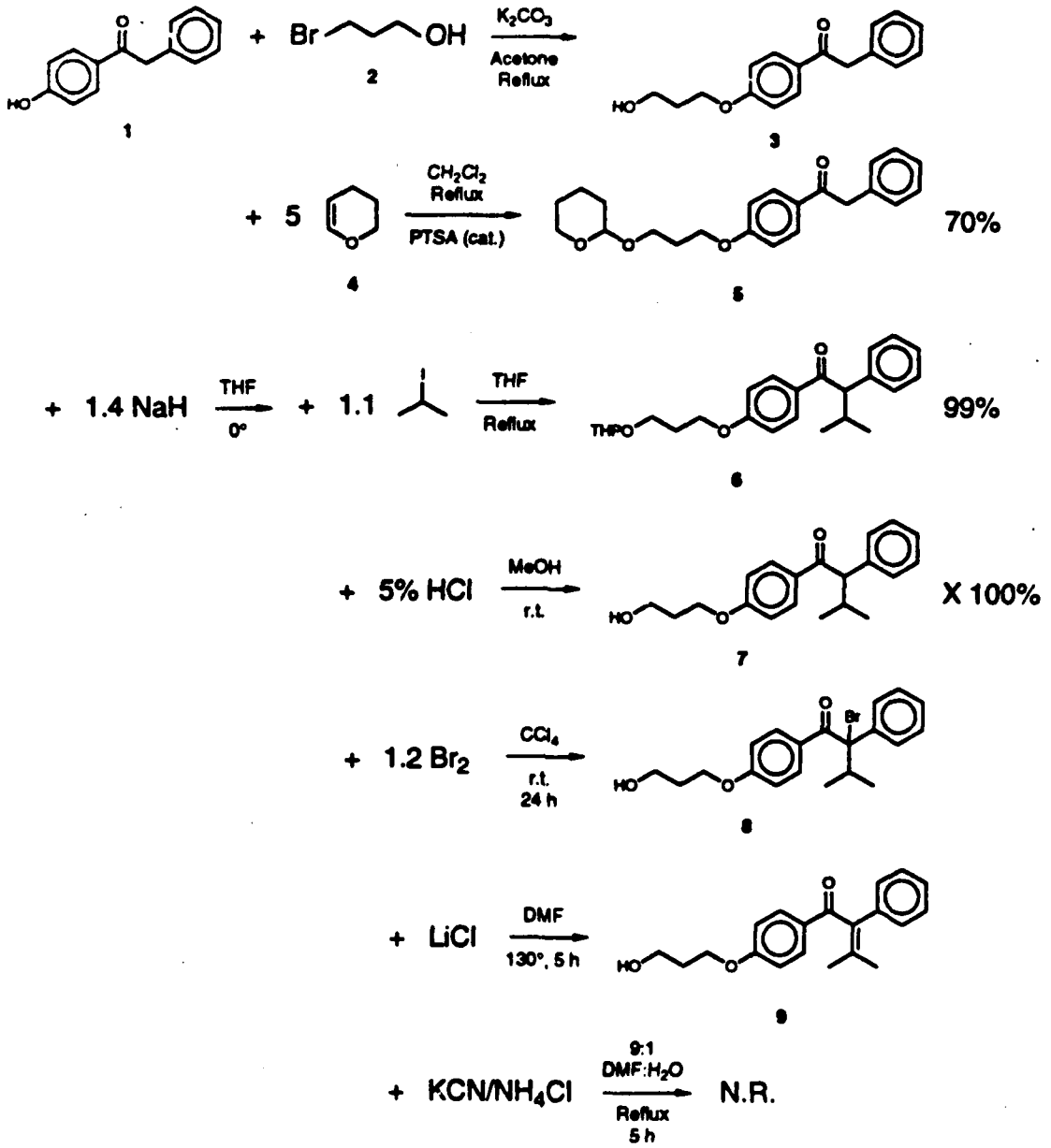

+ Br OH
2
K_2CO_3
Acetone
Reflux
1
3
+ 5
CH_2Cl_2
Reflux
PTSA (cat.)
4
5
70%
+ 1.4 NaH
THF
0°
+ 1.1
THF
Reflux
THPO
6
99%
+ 5% HCl
MeOH
r.t.
7
X 100%
+ 1.2 Br_2
CCl_4
r.t.
24 h
Br
8
+ LiCl
DMF
130°, 5 h
9
+ KCN/NH_4Cl
9:1
$DMF:H_2O$
Reflux
5 h
N.R.
1) Et_2AlCN, 80°
2) TMSCl, py
CN
10
11
very small amounts

CHART I
POTENTIAL CARBOXYLATE CATALYSIS OF CYANIDE ADDITION

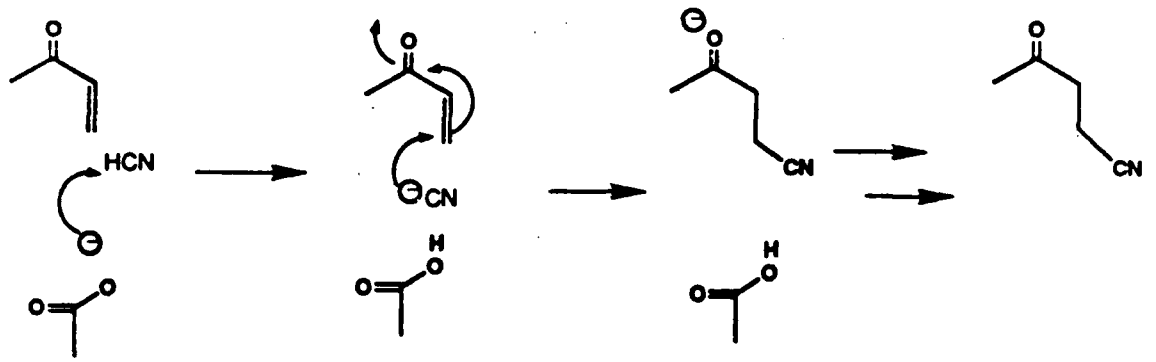
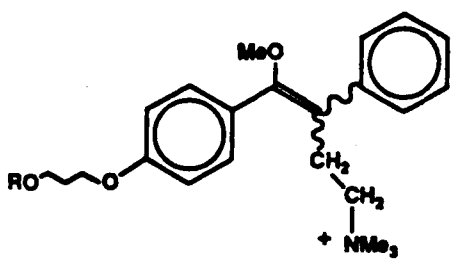
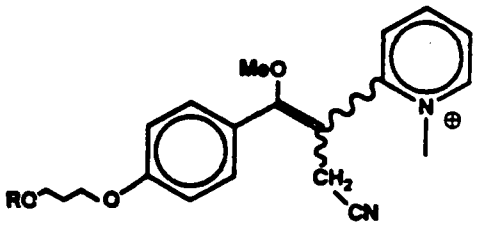


CHART J
"BAIT AND SWITCH" HAPTEN STRUCTURES



1



2

CHART K
ATTEMPTED SYNTHESES OF AMINOALKYL HAPTENS

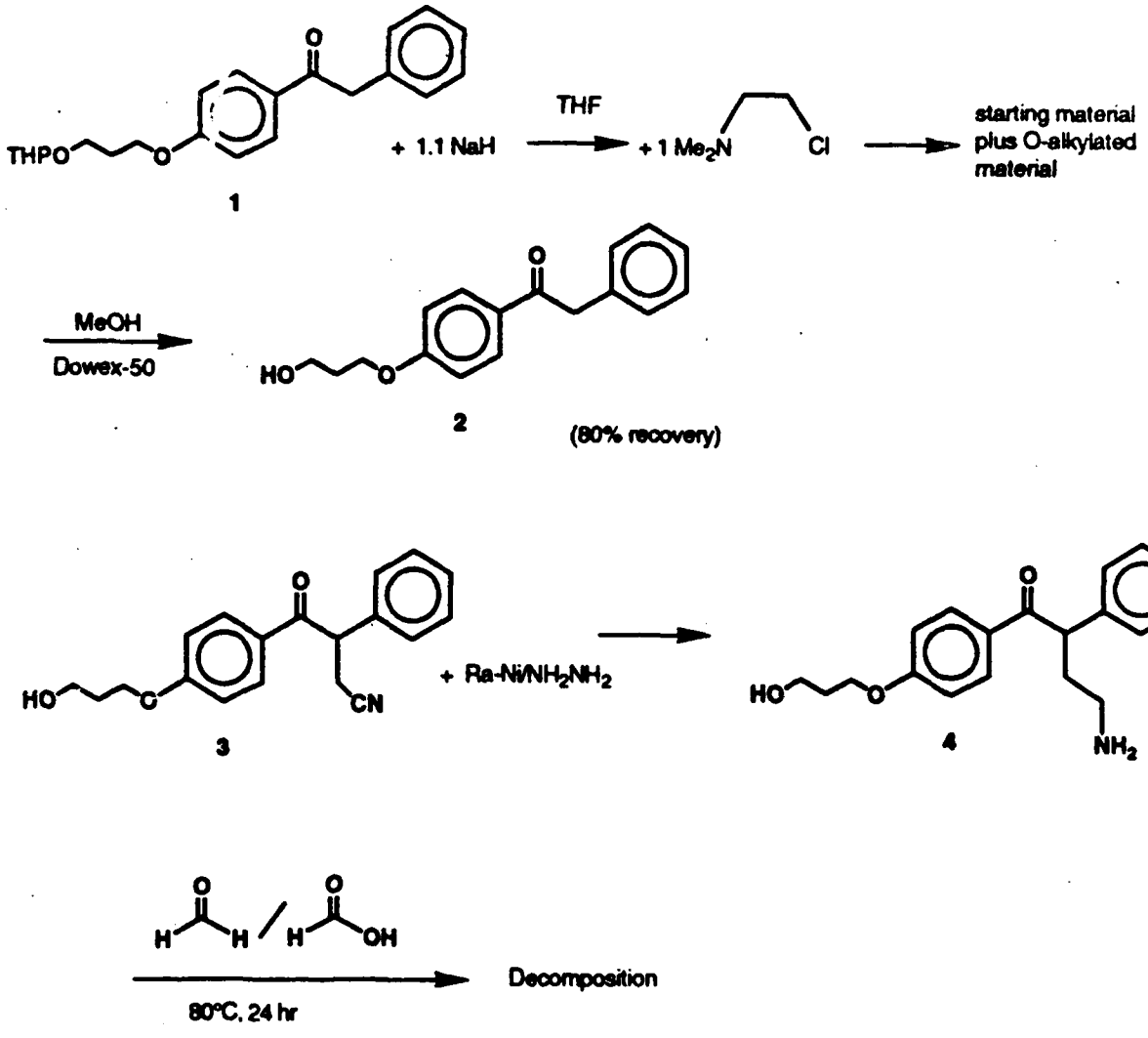
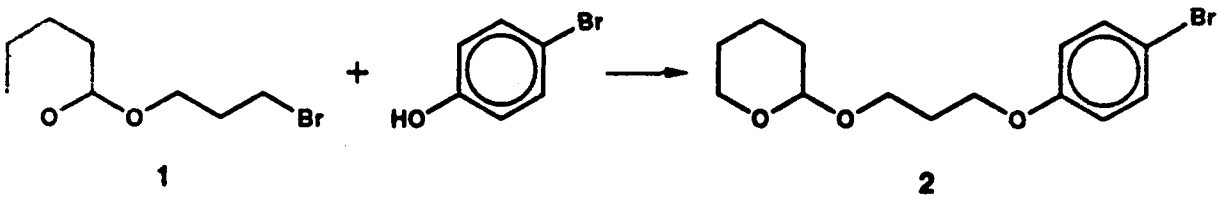
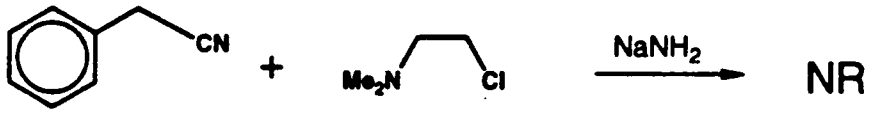
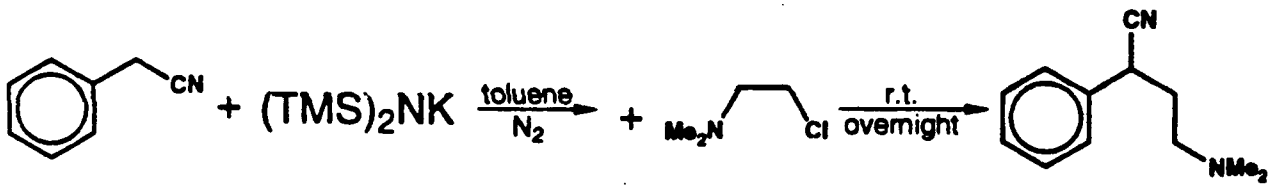


CHART L


Br
O
O
Br
+
HO
Br
O
O
O
Br

1 2


CN
+
Me2N
Cl
NaNH2
NR


CN
+ (TMS)2NK
toluene
N2
+
Me2N
Cl
r.t.
overnight
CN
NMe2

3

CHART M
ROUTE TO A PHENACYLPYRIDINE FOR USE IN HAPTEN SYNTHESIS

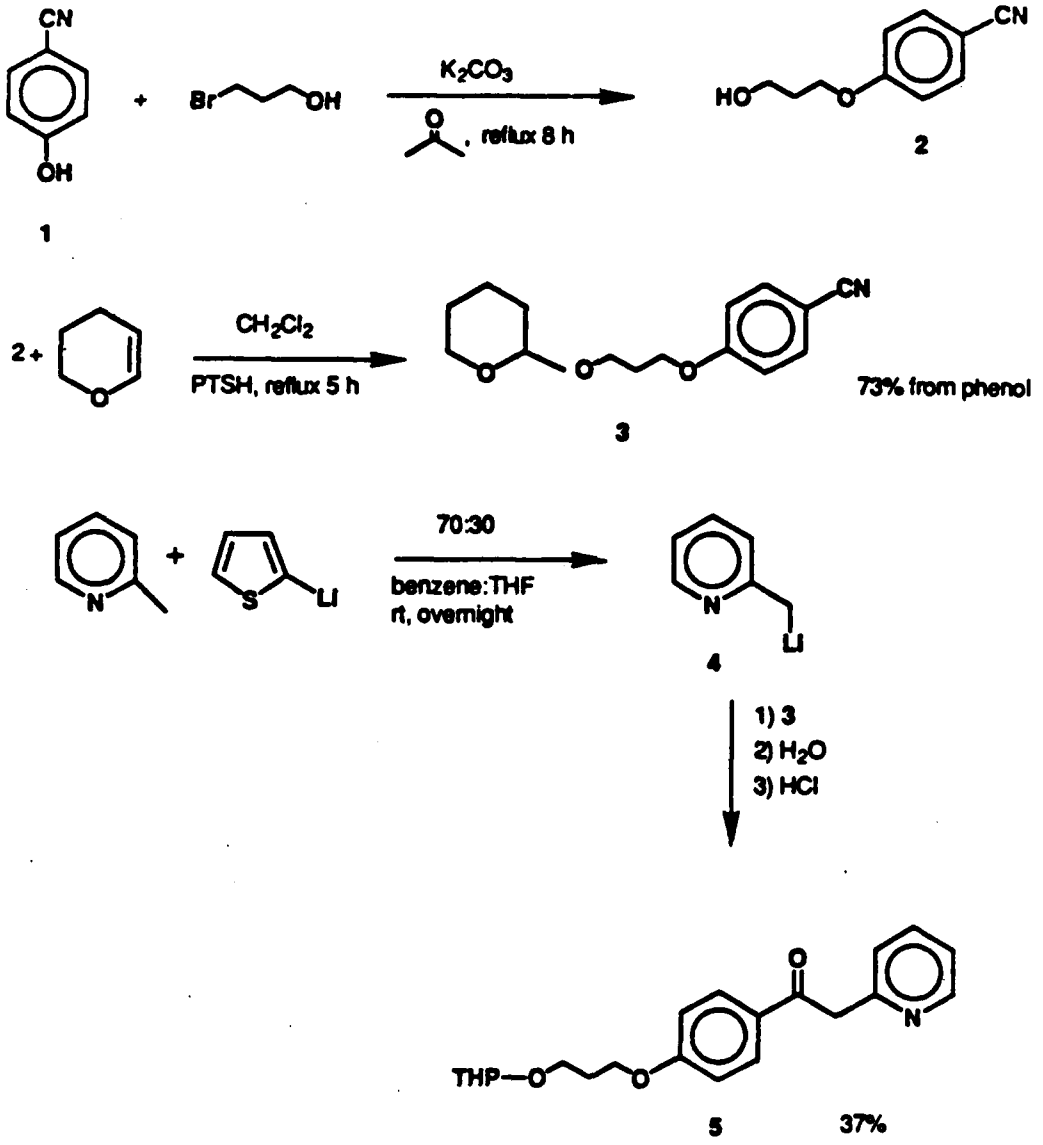


CHART N

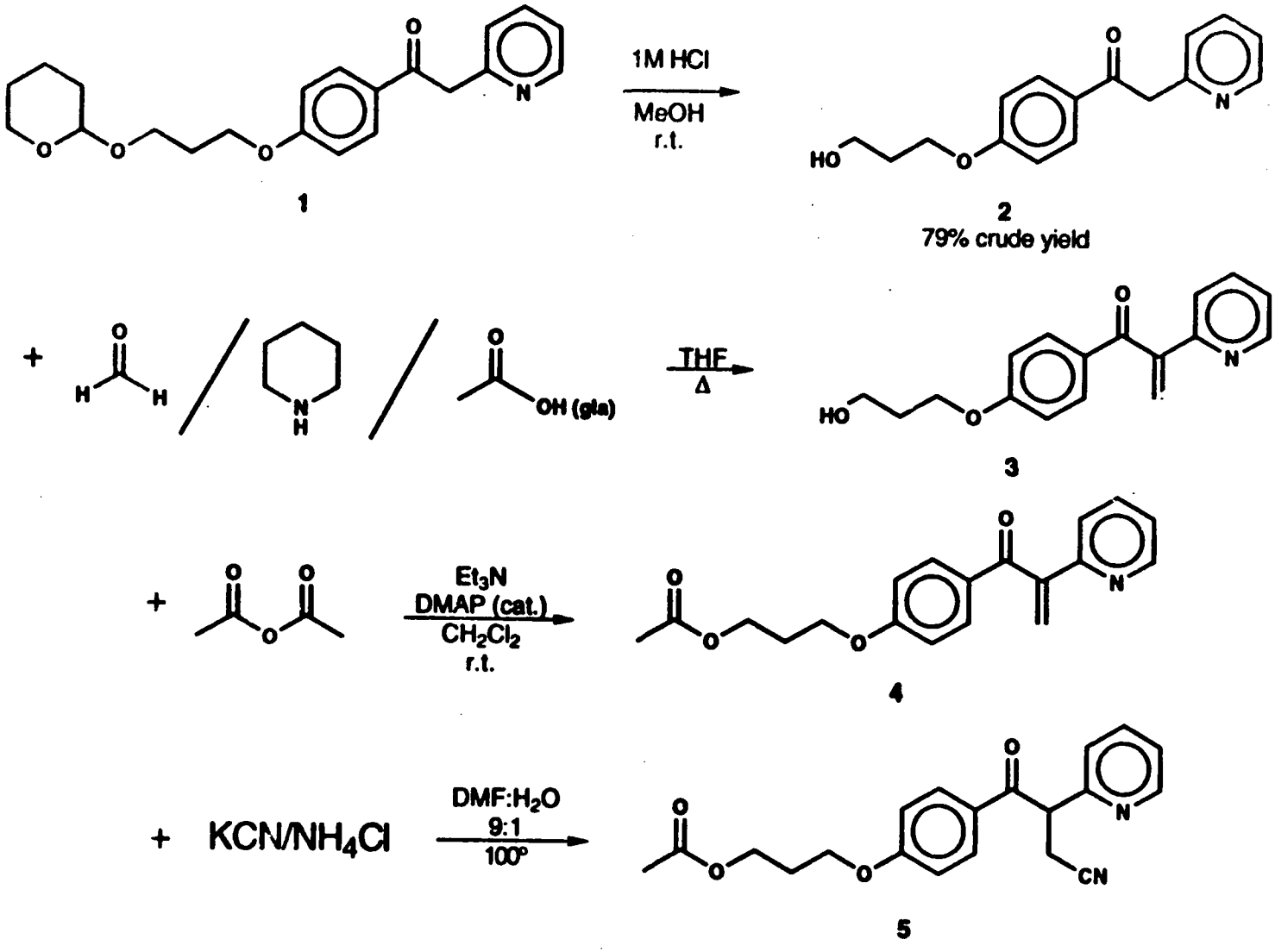
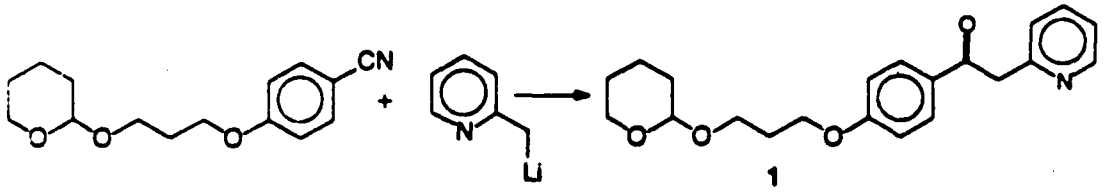
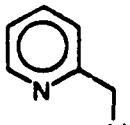
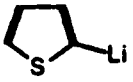
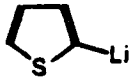
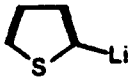
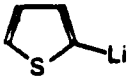
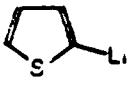

1M HCl
MeOH
r.t.
1
2
79% crude yield
THF
Δ
OH (gla)
3
Et3N
DMAP (cat.)
CH2Cl2
r.t.
4
DMF:H2O
9:1
100°
KCN/NH4Cl
5

CHART O



 Generation	Reaction Conditions	Work-Up	Result
 2:1 ϕH:THF 50°, 3.5 h	Reflux 4 h	water, extract	N.R. recovered nitrile
n-BuLi -20 → r.t. THF, 1 h	70° THF, 3 h	1) H_2O + 1M HCl, 1 h 2) $NaHCO_3$	very little product
0.19 mmol (50 mg)  70:30 ϕH:THF r.t., overnight	80°, 2 h	1) H_2O + 1M HCl, 2 h 2) $NaHCO_3$	37% (SiO_2 column)
2 mmol (522 mg)  70:30 ϕH:THF r.t., overnight	80°, 2 h	1) H_2O + 1M HCl, 2 h 2) $NaHCO_3$	high yield by NMR
1.7 mmol (440 mg)  70:30 ϕH:THF r.t., overnight	85°, 6.5 h	1) H_2O + 1M HCl, 2 h 2) $NaHCO_3$	very little product
4 mmol (1.045 g)  70:30 ϕH:THF r.t., overnight	85°, 6.5 h	NH_4Cl sat.	NR recovered nitrile

3.0 Figures

Figure 1
Effect of Methoxystilbene (Dimethylurethane Derivative TSA) on Antibody Binding

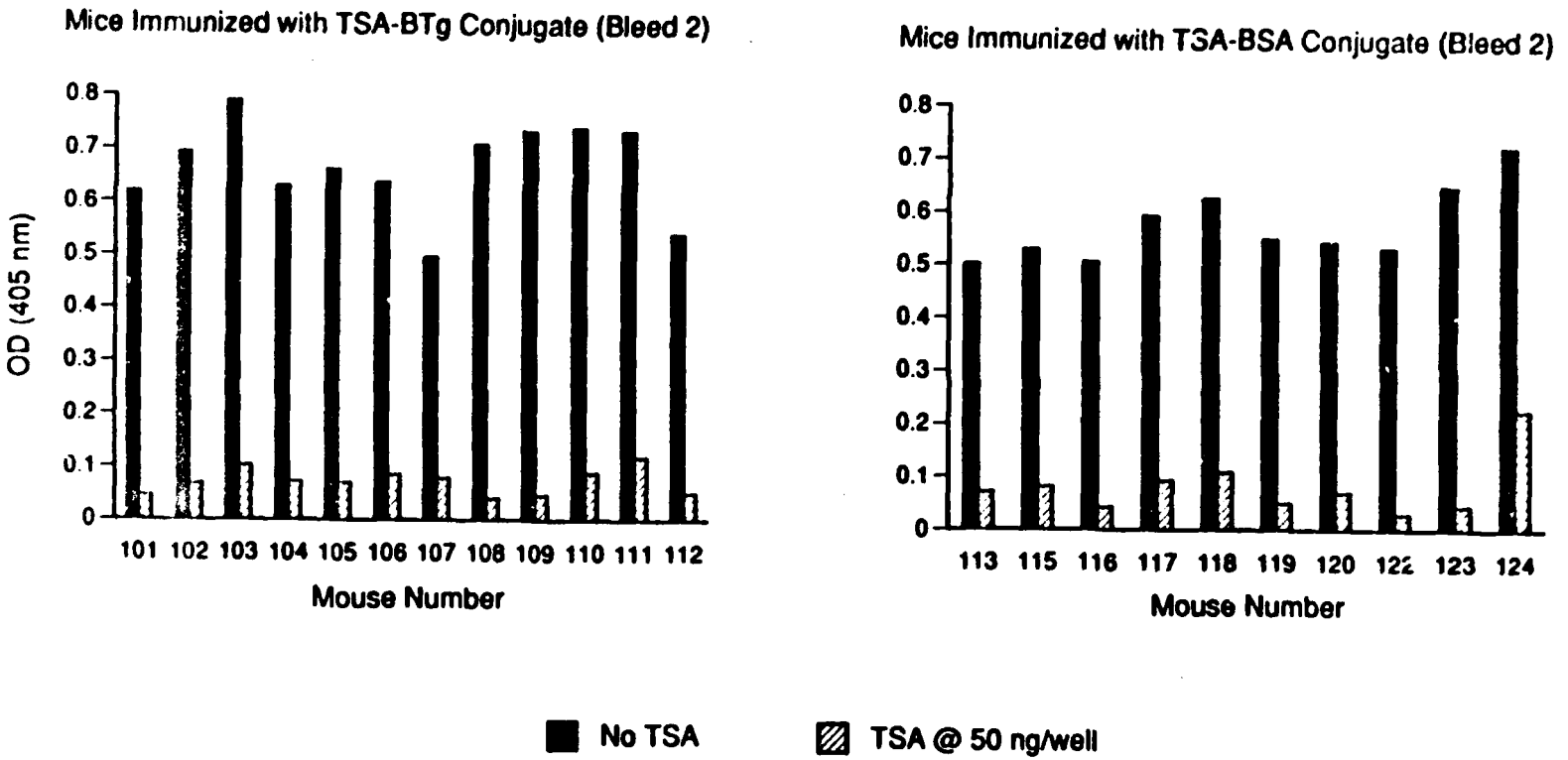


Figure 2

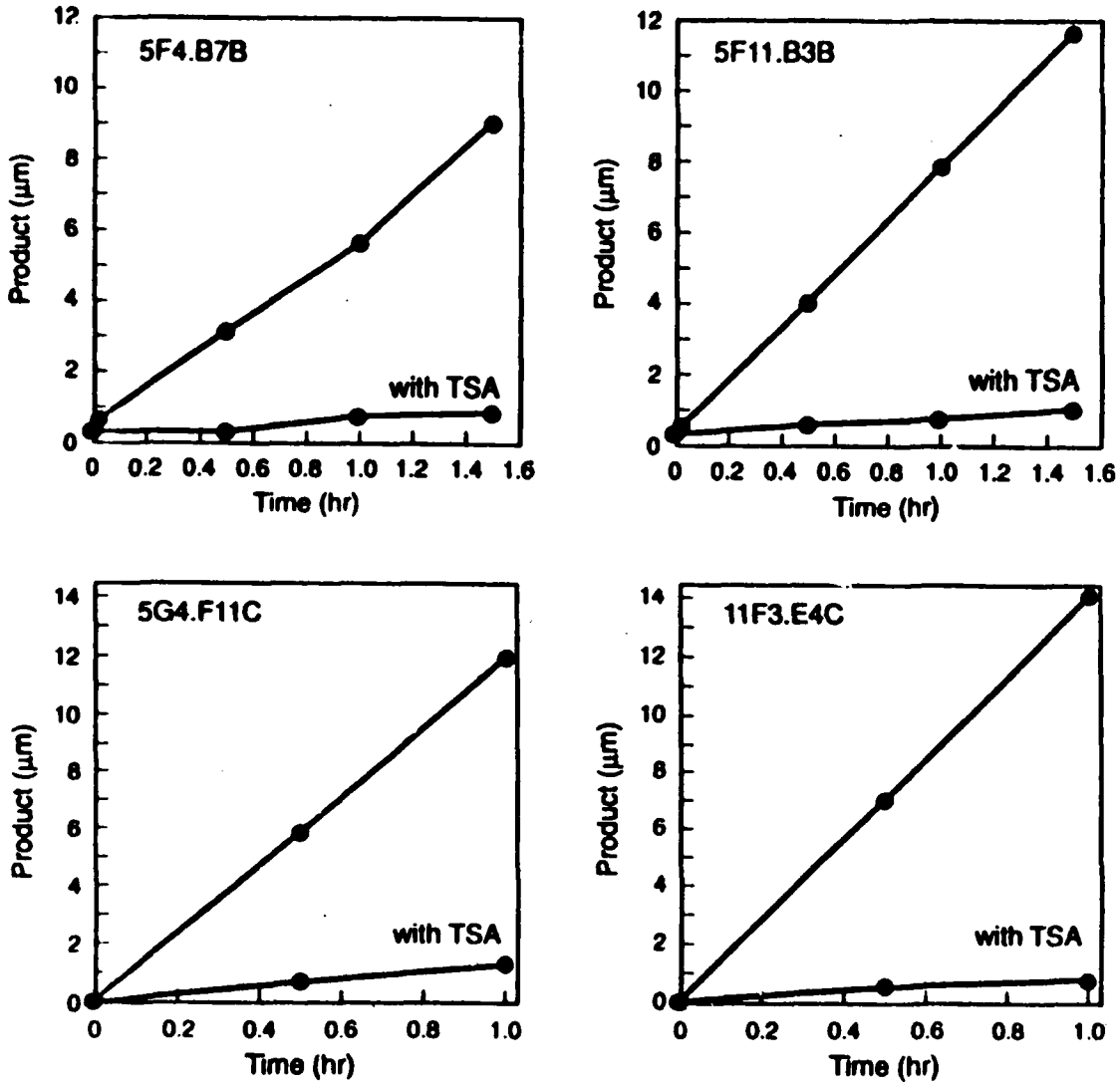

5F4.B7B
with TSA
Product (µm)
Time (hr)
5F11.B3B
with TSA
Product (µm)
Time (hr)
5G4.F11C
with TSA
Product (µm)
Time (hr)
11F3.E4C
with TSA
Product (µm)
Time (hr)

FIGURE 3

Phenyl Plane Angles

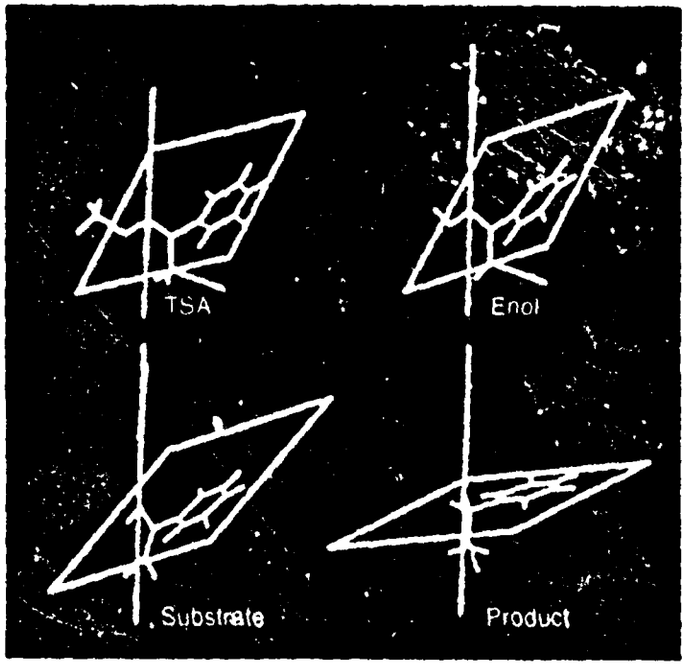
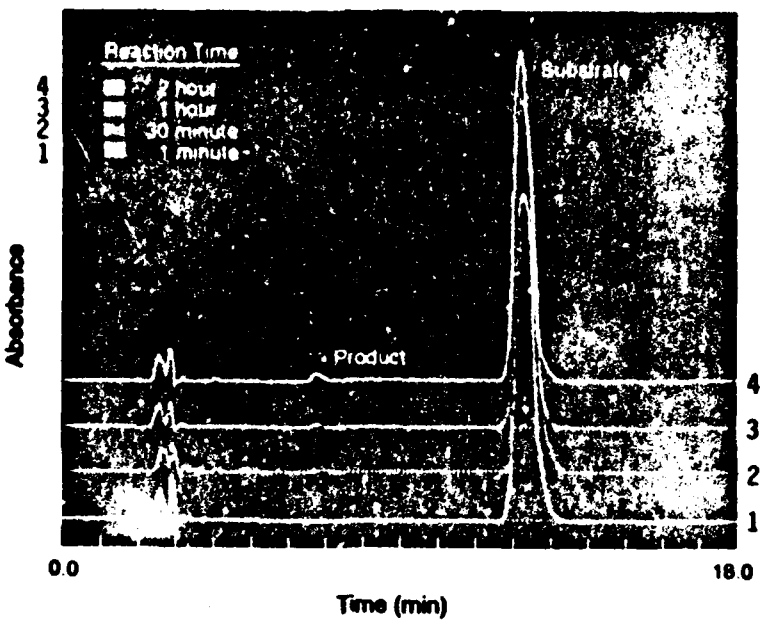


FIGURE 4

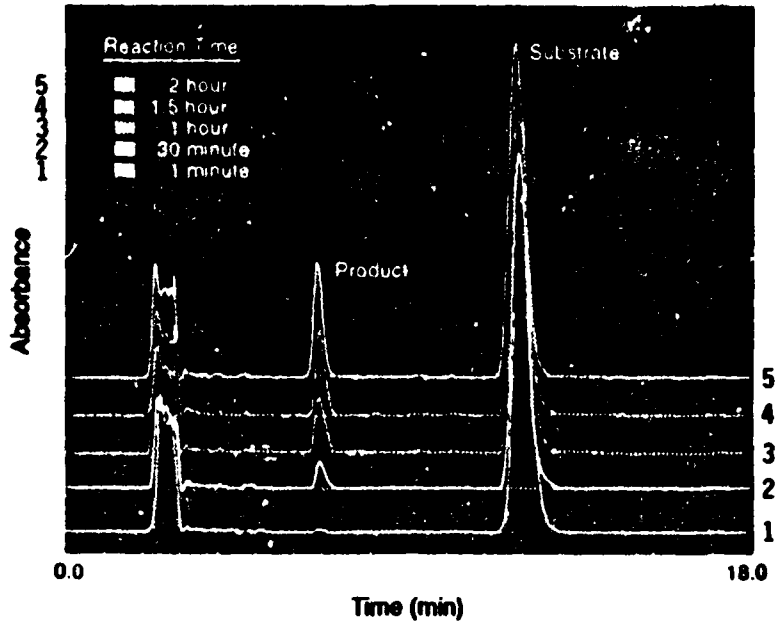
Chromatograms of Background Reaction



Sequential chromatograms of reaction of enone (50 μM) with KCN (5 mM) in absence of antibody (background) in 0.01 M Tris buffer of pH 7.4 at 37 °C.

FIGURE 5

Chromatograms of Antibody-Catalyzed Reaction



Sequential chromatograms of reaction of enone (50 μM) with KCN (5 mM) in presence of antibody 5G4.F11C (20 μM) in 0.01 M Tris buffer of pH 7.4 at 37 °C.

FIGURE 6

Lineweaver-Burk Plots for Enone/Cyanide Reaction

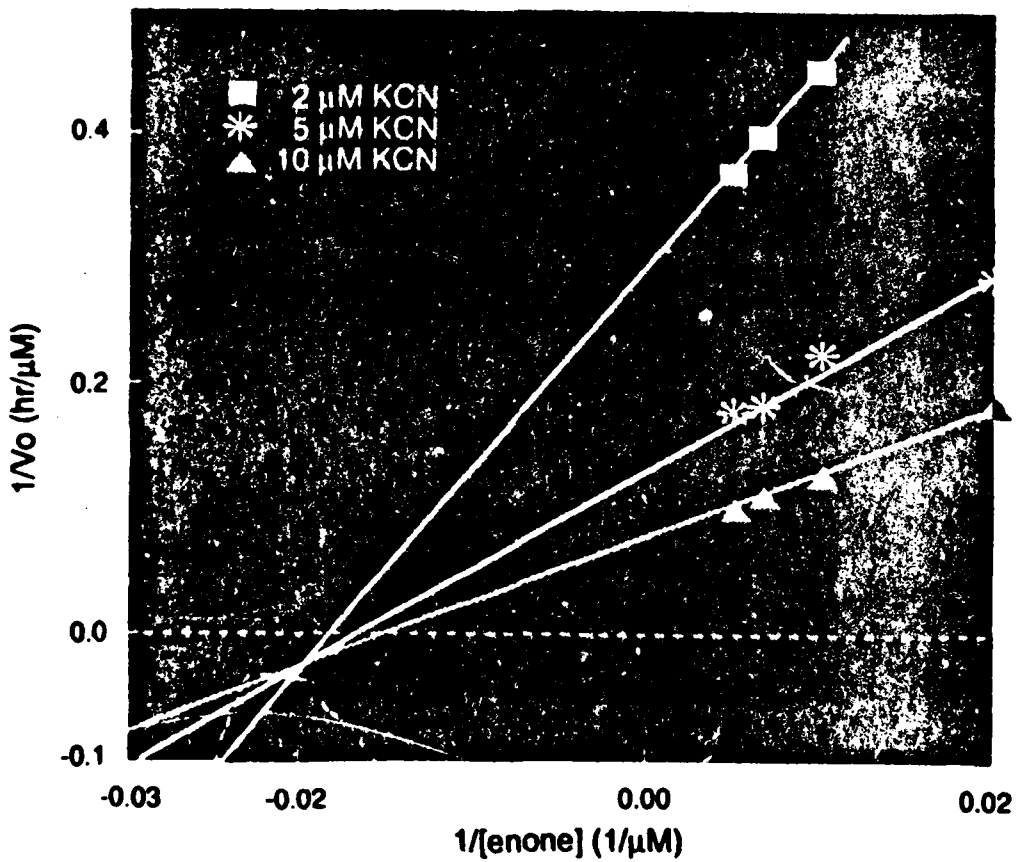


FIGURE 7

Lineweaver-Burk Plots for Cyanide/Enone Reaction

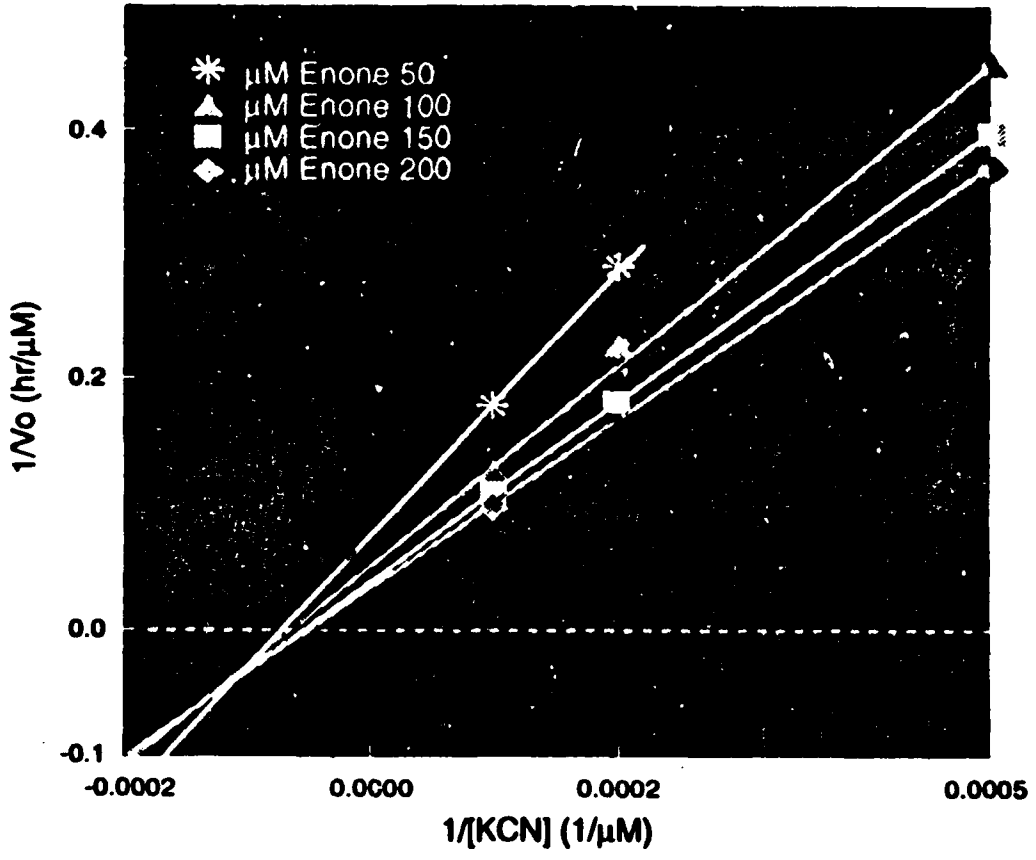


FIGURE 8. **Comparison of actual experimental velocity of reaction catalyzed by antibody 5G4.F11C with rate calculated for a random sequential bireactant model using the parameters determined for the antibody. Line is best fit (y = 1.054 x -0.288; r^2 = 0.9935)**

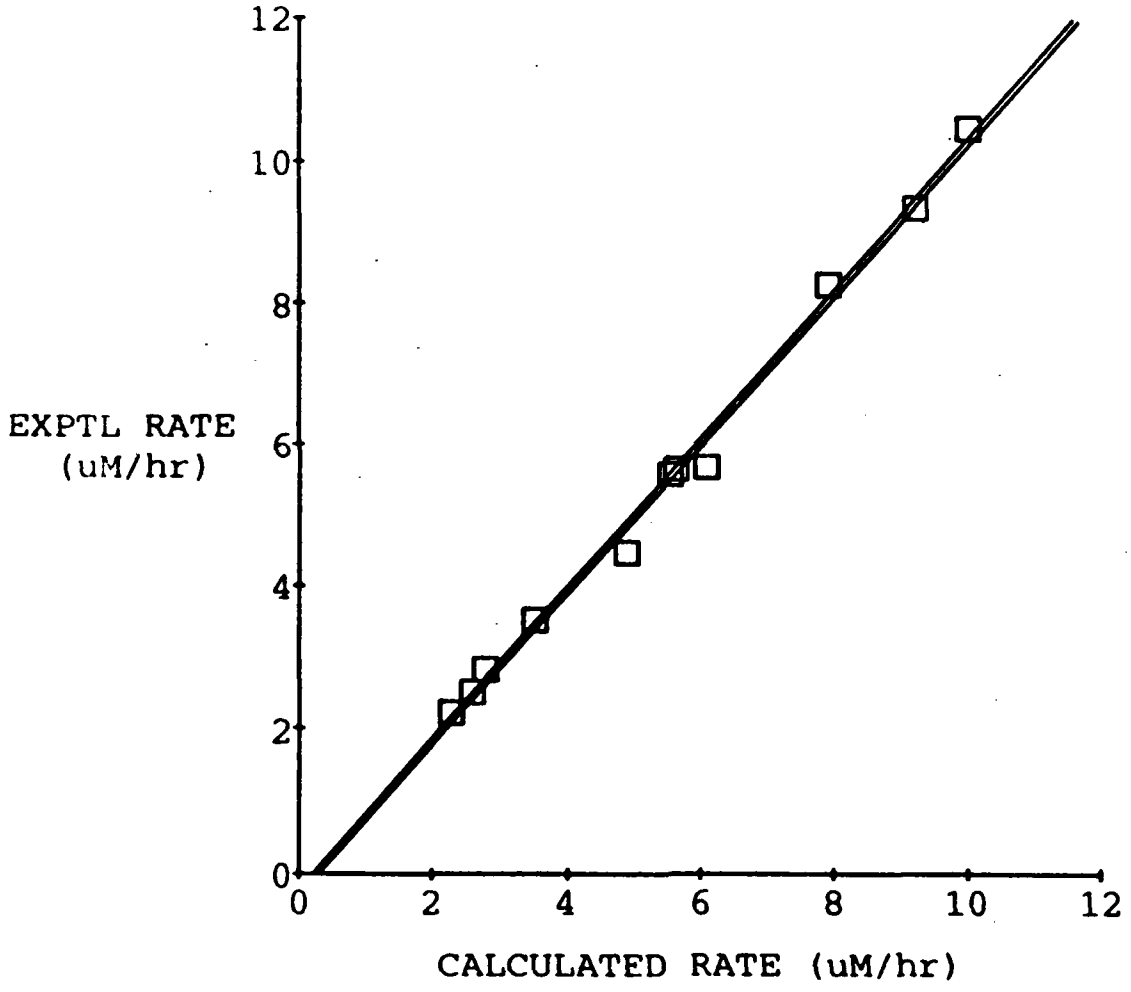


FIGURE 9. **Decrease in KCN with various antibody parameters. For all curves the [IgG] is 50 μM. the top c urve uses the values of K_{enone} (51 μM), K_{KCN} (9600 μM), α (2.5--average of A and B results), and k_{cat} (0.0388 min^{-1}) of the currrent antibody. For the second curve, K_{enone} was changed to 5 μM and k_{cat} to 23.3 min^{-1}. For the third curve, K_{KCN} was further changed to 960 μM. Starting concentrations of enone and KCN were 200 μM and 100 μM, respectively.**

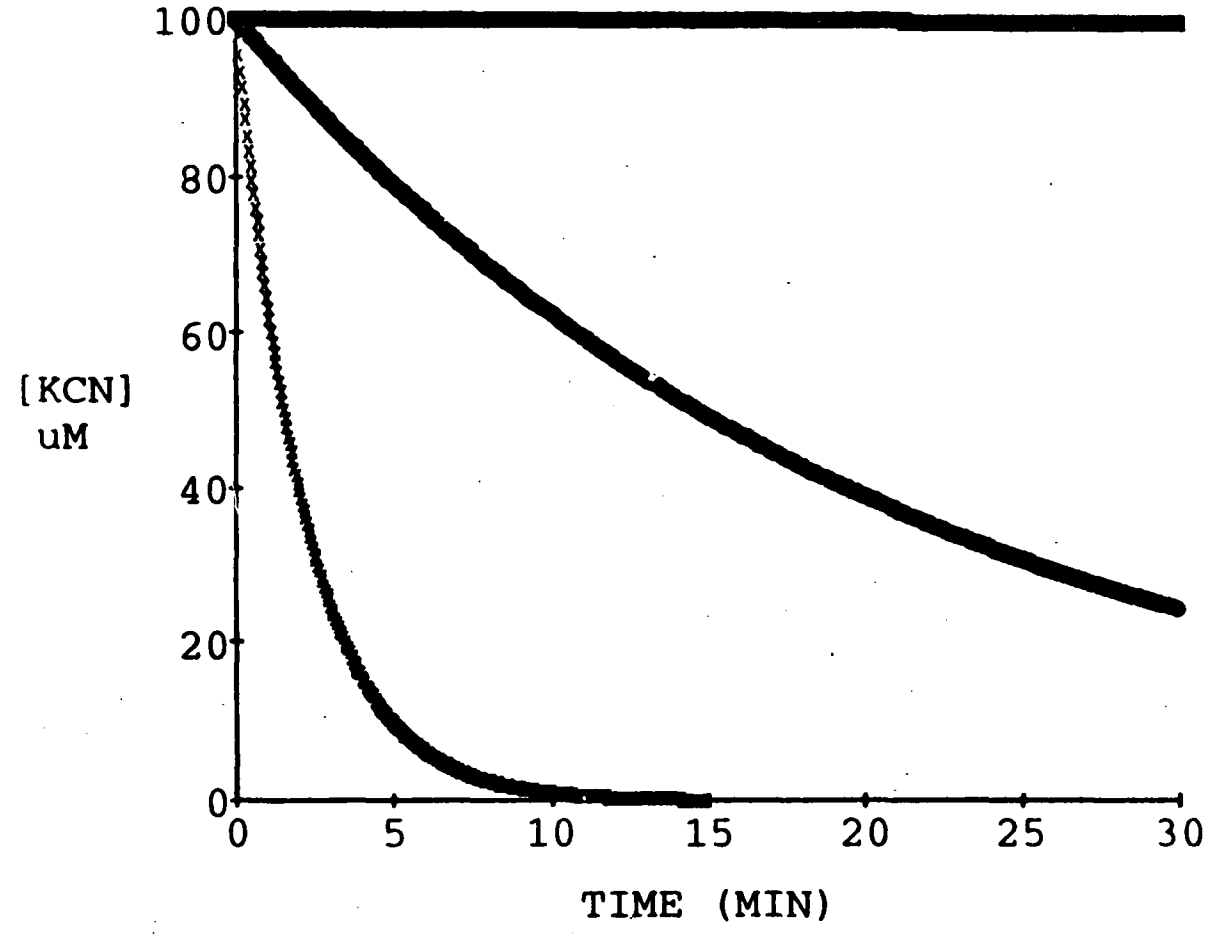
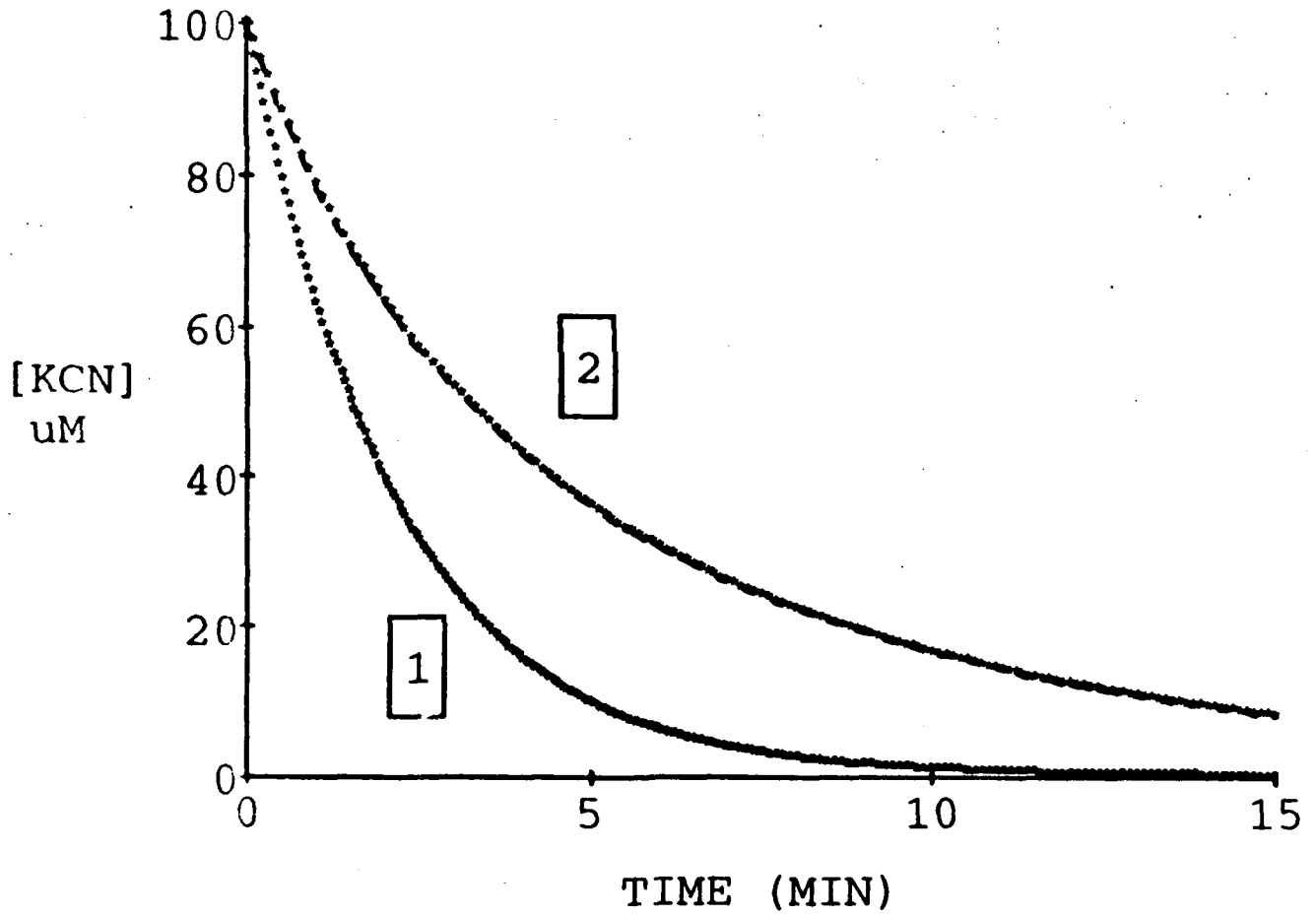


FIGURE 16. **Comparison of simulated antibody catalyzed and uncatalyzed reaction of cyanide with an unsaturated ketone. For curve 1 (antibody catalyzed), the values of the parameters are K_{enone} = 5 μM, K_{KCN} = 960 μM, alpha = 2.5 and k_{cat} = 23.3 min^{-1}. For curve 2 (uncatalyzed), K_{uncat} = 1250 $M^{-1}min^{-1}$. In both cases, the starting concentration of KCN = 100 μM, and the starting concentration of the enone = 200 μM.**



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Cyanide Interactions with Unsaturated Carbonyl Compounds, C. E. Cook, C. C Whisnant, D. B. Miller, B. Radhakrishnan, and S. I. Baskin, Proceedings of the 1991 Medical Defense Bioscience Review, U. S. Army Medical Research Institute of Chemical Defense, August, 1991, pp 261-264.

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Monoclonal Antibodies as Catalysts for Cyanide Removal, C. E. Cook, C. C. Whisnant, D. B. Miller, D. A. Allen, and P. V. Basta. Presented at U. S. Army Medical Research and Development Command 1993 Medical Defense Bioscience Review, Baltimore, MD, May, 1993, IN PRESS.

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No graduate degrees resulted from individuals receiving support from this project.